Predicting malignancy in pre-neoplastic lesions detected during screening for pancreatic cancer.

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by James A. Nicholson.

June 2017

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Acknowledgements

Fate conspired to put me into contact with Mr Louis Vitone, a former EUROPAC fellow, who was working alongside me in the Pancreatic Unit at the Royal Liverpool Hospital. He introduced me to Dr Bill Greenhalf and the illustrious Professor John Neoptolemos who have suffered me as their PhD student for longer than was strictly necessary. I owe both of these men a great deal, I have learnt more from them than they know both in their dealings with people (patients and colleagues alike) and in their determination, resolve and perseverance in supporting research into pancreatic cancer.

All of the previous EUROPAC fellows have contributed to this work in their own way, not least Mr Nathan Howes whose pilot study lit the flame for the eventual pyre of data which this thesis generated. Whilst on the topic of EUROPAC I have to acknowledge the long sufferance, dedication and organisational abilities of Miss Sara Harrison, without whom I would not have survived the first week of my research post, let alone the three and a half years in which we shared an office and the turmoil of life at the cutting edge of pancreatic research.

I have been lucky enough to have travelled to Europe and the United States as well as to cities around the UK to present this work in various forms, this has been generously funded by the Pancreas Biomedical Research Unit, Cancer Research UK, Pancreatic Cancer UK, The Royal Liverpool University Hospital, The European Pancreas Club, The American Pancreatic Association and the Hirschberg Foundation.

In helping me with the lab work I am indebted immeasurably to Dr Li Yan who is perhaps the most patient and wise post-doctoral researcher ever to grace the 5th floor – if she can teach an inpatient surgeon (with borderline ADHD) to perform accurate PCR she must be. Similarly tolerant and kind have been Miss Liz Garner without whom there would be no immunohistochemistry and Miss Katie Bullock who made the ion torrent easy for me by doing all of the hard work.

Professor Fiona Campbell was good enough to spare me time to go through innumerate tissue slides when she had far more important things to do and Professors Sutton and Ghaneh as well as Dr Costello, Mr Halloran and Mr Raraty have provided me with advice along the way, provided patients for the study and been there for support on the days where the gods of PCR weren't benevolent.

Finally whilst I would be the first to acknowledge that this work is a perfect example of the view obtained from the shoulders of giants it has been, nevertheless, a significant undertaking which has been a constant presence in every aspect of my life since August 2010. Suffering along with me and tolerating the intrusion of 'the thesis' into our time together has been my partner and soulmate Andy without whom much would be intolerable and who was always there to console me on the days when I deleted more than I wrote.

J N

Dec 2016

Abstract

Background: Screening is offered to individuals who have an identified increased risk of pancreatic cancer. Such risks may be increased because of a family history or a known genetic mutation which has been shown to confer increased risk. Amongst those being screened intraductal papillary mucinous neoplasm (IPMN) of the pancreas are increasingly common entities being detected incidentally; their risk of malignancy can be as high as 85%. Consensus guidelines exist for the management of these lesions – the morbidity associated with pancreas resection is as high as 50%. We set out to identify a marker which could be used to identify those IPMN which should be resected and those which may be safely observed.

Methods: Individuals were identified from the European Registry of Hereditary Pancreatitis and Familial Pancreatic Cancer (EUROPAC) or patients identified in Liverpool or Hedielberg with cystic lesions and/or pancreatic cancer. Cancer screening was performed by imaging and with molecular analysis (including mutation analysis of *TP53*) of pancreatic juice obtained by endoscopic retrograde cholangiopancreatography (ERCP). Matched tissue sections and frozen sections of pancreatic tissue from 73 patients who underwent resection for IPMN were assessed histologically and for mutations in *TP53* status using a novel limiting dilution Next Generation Sequencing technique. Results were assessed relative to clinical outcomes.

Results:

Amongst those 29 individuals who were screened with ERCP, 11 had IPMN, 7 IPMN with cancer (IPMC) and 3 pancreatic ductal adenocarcinoma (PDAC). The remaining cases were benign neoplastic or inflammatory conditions. Kaplan-Meier survival analysis at 5 years found that the presence of p53 mutation in the tissue was a better prognostic marker of survival than the histological diagnosis alone ($p=0.0152$ vs. $p=0.0819$). Sensitivity and specificity of p53 mutation as a predictor of survival was calculated as 0.89 and 0.95 respectively. There was 100% correlation between the p53 mutational status of the resected tissue and the pancreatic juice obtained at ERCP.

When ERCP was assessed as a method for screening, however, there was found to be an unacceptably high incidence of post-ERCP pancreatitis (PEP) 7 cases of PEP in 16 ERCPs (44%). This rate was shown to be significantly reduced to 15% (6/40) with the use of pancreatic stent and diclofenac, but the overall prevalence of PEP was 23.2% over 14 years. There were no cases of PEP amongst those individuals being screened because of hereditary pancreatitis.

Of 27 IPMN cases with frozen tissue 23 individuals had *TP53* mutations. Seven cases died of pancreatic cancer after resection. Kaplan-Meier survival analysis revealed that one mutation p.L264R predicted survival regardless of histology (p=<0.0001). The mutation was present in 6 of the 7 cases who died and in none of those who survived to 5 years. Mutation specific PCR was used to validate results showing that p.L264R discriminated between survivors and IPMN cases who died of cancer (AUC = 0.79).

Conclusions: IPMN continues to cause concern and uncertainty among those individuals being screened for cancer who are largely well and asymptomatic. The p.L264R mutation could be used to differentiate those IPMN which result in poor survival to facilitate potentially curative surgery. The mutation may be present in pancreatic juice which can be collected endoscopically as a screening tool. The use of prophylactic measures to reduce PEP may be considered sufficient to bring the risk of complications to an acceptable level when compared to the relative certainty of prognosis afforded by a positive test for p.L264R.

CHAPTER 1

BACKGROUND

1.1 - Overview

That pancreatic cancer is a terrible disease need not be overstated; the symptoms often present too late and even with surgical resection and adjuvant chemotherapy survival rates are abysmal. One method utilised to try to improve the odds for patients is that of selecting high risk individuals in whom screening can be undertaken with the aim of early detection and cure.

Identifying such individuals is relatively easy - the risk factors and associated mutations for some forms of pancreatic cancer are well known - what is not well known, however, is the best method of early detection. Such screening investigations invariably carry risk to the patient and also present the uncomfortable clinical quandary of the 'incidentaloma'; a lesion which is likely to be benign but once found cannot lightly be ignored. Commonly these lesions are cystic in nature, for example IPMN.

The ability to separate those lesions which harbour sinister potential from those which do not is a 'holy grail' of sorts for the world of pancreatologists. In doing so we would be able to spare patients unnecessary (and potentially fatal) operative procedures and focus our attentions on those who would benefit from surgery and other treatments.

This thesis outlines my work, undertaken at the Royal Liverpool University Hospital in conjunction with the University of Liverpool between 2010 and 2014 to improve the safety and effectiveness of screening for pancreatic cancer among high risk individuals.

It also describes the discovery of a mutation of *TP53* previously unreported in IPMN which was detected within resected cystic lesions of the pancreas, the presence of which shows a significant correlation with poor overall survival.

Novel data include:

1.) Data to support the notion that a mutation of the *TP53* gene is more reliable at predicting malignant potential than histological diagnosis.

2.) Data relating to a 12 month collection of secretin stimulated pancreatic juice from the duodenum of 120 patients with benign and malignant conditions of the pancreas.

3.) The results of screening investigations performed on 172 individuals identified as being high risk for pancreatic cancer.

4.) A description of a novel next-generation sequencing method used to analyse *TP53* with increased accuracy and depth.

5.) Evidence that a single mutation of *TP53* can be used to predict mortality in IPMN of the pancreas.

This work also includes data previously published wholly, or in part, wherein I am the first author:

- Nicholson JA, Greenhalf W, Jackson R, Cox TF, Butler JV, Hanna T, et al. Incidence of Post-ERCP Pancreatitis From Direct Pancreatic Juice Collection in Hereditary Pancreatitis and Familial Pancreatic Cancer Before and After the Introduction of Prophylactic Pancreatic Stents and Rectal Diclofenac. Pancreas. 2015; 44(2): 260-5.

1.2 - Pancreatic Cancer

Pancreatic cancer is the 10th most common cancer in the UK, it has a male to female ratio of 1:1 and there are 13.9 new cases per 100,000 population every year.[1] The age-standardised rate is 9.2 per 100,000.[1] Five year relative survival rates for pancreatic cancer are the lowest of the 21 common cancers in England.[2] The relative current survival at 5 years is 3.6% and 3.8%, for men and women respectively and 2.9% and 2.7% at 10 years.[3] It should be noted however, that these rates include cases of pancreatic cancer which have been treated **and** those which have not.

Whilst there can be varying forms of malignant neoplasms which affect the pancreas, the commonest by far is pancreatic ductal adenocarcinoma. There are many different forms of pancreatic cancer, which can be grouped into those which arise in the exocrine pancreas and those which arise in the endocrine pancreas. A third rare subgroup of pancreatic cancer consists of pancreatic lymphoma.[4]

Table1: Overview of 5-year survival and incidence of pancreatic neoplasms.

Neoplasm	% of Pancreatic Neoplasms	5-year Survival
Pancreatic Ductal Adenocarcinoma (PDAC)	90%[5]	3-5%[6, 7]
Intraductal Papillary Mucinous Neoplasm (IPMN)	3%	varies depending on progression to malignancy.[8]
Mucinous Cystadenoma (MCA)	<1%[9]	33%[10]
Serous Cystadenoma (SCA)	2%	50-70%
Acinar Cell Carcinoma	1%	<5%
Primary Pancreatic Lymphoma	0.5%[4]	45%
Non-functioning Islet Cell Tumour	2%	50-60%
Functioning Islet Cell Tumours:	0.2% of which:	33%
	Gastrinoma (46%) [11]	-
	Insulinoma (27%)[12]	-
	Glucagonoma (17%)[13]	-
	VIP-oma (10%)[14]	-
	Somatostainoma (no data)	-

The obvious question becomes – why the abysmal survival rate when this is clearly a common cancer? The answer is multi-factorial.

Diagnosis

The symptoms of pancreatic cancer are notoriously non-specific, a recent qualitative analysis of interviews with 32 pancreatic cancer patients and 8 relatives confirmed that not only is there no true pathognomonic sign or symptoms, but any symptoms which do occur may well be intermittent for up to 12 months prior to the diagnosis.[15] The symptoms which were most often reported included: pain in the upper abdomen; pain in the back or shoulder and indigestion or heartburn.[15] At presentation with exocrine pancreatic cancer, the most frequent symptoms were lethargy (86%), anorexia (85%), weight-loss (85%), abdominal pain (79%), and choluria (59%).[16] One Italian study of 170 patients treated for pancreatic cancer concluded that time of diagnosis to surgery negatively correlated with survival and that the time from presentation to diagnosis was shorter when the presenting symptom was pain compared with weight loss.[17] The inference here is that survival depends on prompt diagnosis.

The risk factors for pancreatic cancer are generally well known; in 2001 Debra Silverman published an epidemiological review of the aetiological factors associated with pancreatic cancer. These have been reproduced in Table 2.[18] This work is currently being updated as part of the PanGen-EU study.

Table 2: Risk factors for PDAC.[18]

Risk factor	Odds Ratio for Pancreatic Cancer (95% CI)
Family history (≥ 1 case of pancreatic cancer)	3.2 (1.8 – 5.6)
Heavy drinking (>85.5 units per week)	2.2 (0.9 – 5.6)
Cigarettes (>20 per day)	2.0 (1.6 – 2.9)
Obesity (BMI ≥ 35)	1.5 (0.9 – 2.5)
Diabetes Mellitus for >10yrs	1.5 (1.01 – 2.2)

Even if the patient presents promptly with sufficient symptoms to alert a doctor – the next hurdle to be cleared is actually making the diagnosis. Broadly speaking a diagnosis is implied by history and examination, suggested by investigations such as imaging and serum analyses and confirmed by histology or cytology.

Current investigations in routine use which are aimed at diagnosing pancreatic cancer consist of serum biomarkers (CA19-9) and imaging techniques such as multi-slice computed tomography (CT), endoscopic ultrasound (EUS), and magnetic resonance imaging (MRI).

Carbohydrate antigen 19-9 (CA19-9) was first identified as a tumour marker for colonic cancer in 1981, however at that point only 2 patients with pancreatic cancer had been included in the first study.[19] In 2007 a systematic review of published data on CA19-9 concluded that the median sensitivity was 79% (95% CI: 70-90%) and median specificity was 82% (95% CI: 68-91%).[20] More recently a meta-analysis of 14 studies has suggested that the addition of CA242 could increase the specificity even further.[21] A further problem for CA19-9 is that it is shown to be raised in non-malignant obstruction of the biliary tree,[20] also patients who are Lewis Antibody negative ($Le^{a-/b-}$) (5-7% of the population in Japan) do not express CA19-9.[22, 23]

CT is the most widely used imaging modality in the developed world when it comes to making a diagnosis of pancreatic cancer, and is especially useful in the staging of PDAC.[24-27] The sensitivity of lesion detection with CT is 93-100% in various series whilst the positive predictive value for tumour detection is greater than 90% although this obviously depends upon the screened population.[24, 28] Unfortunately, approximately 25% of patients considered to have resectable disease on CT are found to be unresectable intra-operatively.[28] One of the greatest difficulties for a radiologist reporting a CT of the pancreas is to differentiate between chronic pancreatitis and adenocarcinoma.

MRI is now generally reserved for assessment of fluid-filled cystic lesions of the pancreas but the addition of dynamic gadolinium enhanced MRI scanning has made MRI comparable to CT in some studies.[26]

EUS was initially considered to be superior to both CT and MRI prior to refinements and enhancements in their use.[27] It has been shown to be more accurate than CT in tumour size determination and in assessing nodal involvement but is inferior for assessment of loco-regional extent, vascular invasion, distant metastases and resectability.[29]

As well as chronic pancreatitis, numerous other 'benign' conditions may be mis-diagnosed as malignant or allocated into the 'uncertain' category where resection is often the only way to achieve a definitive diagnosis. Such asymptomatic lesions (or "incidentalomas") are now known to comprise between 6 to 23% of all pancreatic resections for any cause.[30-32]

Treatment

Initially the treatment of pancreatic cancers must be separated into those which are deemed inoperable and those which are felt to be amenable to attempted curative resection. The treatment of patients who have localised advanced disease, metastases or a poor performance status is directed at symptom control.[33]

At present tumours which are felt to be resectable usually progress to the operating theatre as soon as is practical – neoadjuvant chemotherapy or radiotherapy is only indicated in the context of a clinical trial because PDAC is highly resistant to conventional methods of cytotoxic treatment and radiotherapy.[34-37] Recent prospective cohort studies of neoadjuvant treatment have shown a modest survival benefit but the duration of treatment is still contentious.[38, 39]

Surgical resection of the pancreas, or part thereof, is incredibly delicate surgery and international consensus now agrees that such procedures should only be undertaken in supra-regional centres with a high volume of procedures being performed annually.[40-44] Despite advances in surgical technique the published figures for morbidity and mortality are generally around 46% and 3.8% worldwide. Table 3 shows pooled mortality and morbidity figures for pancreatic resection identified in a meta-analysis by Chen, *et. al* in 2013.[45]

Once a patient has undergone their (hopefully successful and uncomplicated) surgical intervention there is usually a requirement for adjuvant chemotherapy – radical resection alone will result in a 5-year survival of only 10%.[40] As a consequence systemic chemotherapy is offered – trials have shown that there is no survival benefit between adjuvant chemo-radiation and systemic chemotherapy

alone.[35] The results from two large randomised trials show that adjuvant systemic chemotherapy will increase the 5-year survival from 10% to 21-29%.[46-48]

Table 3: Morbidity & mortality rates for selected studies of pancreatic resections. [a]Morbidity includes: pancreatic fistulae; anastomotic leak; post-operative bleeding; return to theatre within 30 days; surgical site infection; pulmonary or deep vein thrombosis.

Study	Country	n	Morbidity Rate (%)[a]	Mortality Rate (%)
deCastro et.al. (2009)[49]	The Netherlands	652	50.9	3
Debinska et.al. (2011)[50]	Poland	65	32.4	-
Gallacher et.al. (2011)[51]	UK	81	54.1	-
Khan et.al. (2003)[52]	UK	50	46	4
Knight et.al. (2010)[53]	UK	99	40.9	-
Pratt et.al. (2008)[54]	USA	326	53.1	1.2
Tamijmarane et.al. (2008)[55]	UK	241	-	7.8
Zhang et.al. (2009)[56]	China	265	39.6	3.8
TOTALS / MEDIAN		**1997**	**46**	**3.8**

Summary

Although the overall picture for surviving pancreatic cancer is poor, survival rates are increasing and with a combination of surgical resection and adjuvant chemotherapy a 5-year survival of 29% is obtainable. The real problem is identifying those patients with cancer early and separating those patients with potentially malignant disease from those with benign conditions – as I have shown, undergoing a resection of the pancreas still carries a risk to life and *ideally* should never be undertaken unless it is absolutely necessary.

1.3 - Cystic Lesions

Cystic lesions of the pancreas present a difficult clinical quandary to pancreatologists – to operate or not to operate. Some of these cystic neoplasms have the potential for malignancy and thus timely resection can prevent pancreatic cancer. As shown, resection of the pancreas (or part thereof) carries significant risk of morbidity and mortality (28%-50% morbidity). [30, 31, 57, 58] As mentioned previously, asymptomatic pancreatic lesions or "incidentalomas" are now known to comprise between 6-23% of pancreatic resections for any cause and the prevalence of cystic asymptomatic pancreatic lesions is now reported to be between 1.2 and 2.6%.[31, 32, 44, 57, 59, 60]

The detection of these incidental lesions is becoming increasingly burdensome to regional pancreatic multidisciplinary teams (MDT) both in terms of the number of patients which need to be reviewed but also in terms of the regular surveillance imaging required once such a lesion has been found.

IPMN

In reality cystic neoplasms represent only a minority of pancreatic cystic lesions as most 'cysts' in the pancreas are pseudocysts (histological examination of a pseudocyst reveals a cyst surrounded by inflammatory tissue with no epithelial lining).[61] Since malignancies most commonly arise from cysts with mucinous contents, focus has shifted to a particular sub-type of cyst which harbours malignant potential: intraductal papillary mucinous neoplasm (IPMN).[62, 63] Although the true potential for malignancy depends upon the subtype of IPMN the overall incidence of IPMN associated malignancy is estimated to be 50%.[64] First reported in 1982, IPMN was recognised as a distinct pancreatic neoplasm by the World Health Organisation in 1996.[65]

IPMN is defined as a grossly visible, non-invasive, mucin producing, predominantly papillary or rarely flat epithelial neoplasm arising from the main pancreatic duct or branch ducts, with varying degrees of duct dilatation.[61] Put more simply an IPMN is an intraductal proliferation of mucin-producing cells

arranged in papillary formations.[66] IPMN can be classified according to various parameters based upon:

1) Their degree of cyto-architectural atypia.
2) The morphology of the neoplastic epithelium.
3) The location within the pancreas (main duct/side branch).
4) The expression of various mucins (e.g. MUC1, MUC6).
5) Their size.[67-70]

At the simplest level IPMN can be categorised into: Main Duct (MD) IPMN meaning that the cavity of the mucinous lesion has arisen from the epithelial lining of the main pancreatic duct; or side-branch (SB) IPMN where the neoplasm is found in one of the smaller side-branch sub-divisions of the pancreatic ductal tree.[66] In reality it is not unusual for IPMNs to extend microscopically several centimetres beyond the grossly visible lesions.[70] Essentially current opinion among surgeons asserts that MD-IPMN should proceed to surgical resection on the basis that the risk of malignancy among MD and mixed-duct IPMN is 60-70%.[71] Often the differentiation between MD, SB and mixed-duct IPMN can be made on imaging.

Histologically there are two classification of IPMN in use – the first is related to the observed degree of nuclear atypia and the second is based upon the morphology of the epithelium.

There are three main groups of cyto-architectural atypia:[72]

1.) Adenoma or low-grade dysplasia.

2.) Borderline or moderate dysplasia.

3.) *In-situ* carcinoma or high-grade dysplasia.

It is perhaps obvious that the higher the degree of dysplasia the more likelihood of the neoplasm invading the surrounding tissue to become malignant or an invasive cancer.

The other sub-classification of IPMN is made based upon the histological appearance, Table 4.[68]

Table 4: Histological sub-types of IPMN.

IPMN sub-type	Histological description[66]	Incidence[73]
Gastric	Neoplastic epithelium resembling gastric foveolae with short finger-like papillae, small pyloric-type glands are often present at the base.	39.3%
Intestinal	Long villous projections lined with mucin-rich columnar cells (similar to colonic adenoma).	36.1%
Pancreatobiliary	Complex branching papillae which are lined with cuboidal cells containing little mucin.	19.7%
Oncocytic	Neoplastic epithelial with abundant eosinophillic cytoplasm but usually little mucin and line the papillae in several layers which can combine to form complex aggregates.	4.9%

Recently a further, 5th sub-type has been proposed: 'intraductal tubulopapillary neoplasm', however at the time of writing the number of references to this in peer-reviewed journals is very low.[74]

The relative incidence of these sub-types is demonstrated in the table above and this correlates somewhat with the progression to invasive malignancy. A review of IPMN cases in one large specialist centre in Japan concluded "Intraductal papillary-mucinous neoplasm of the gastric and intestinal types may have less malignant potential than that of the pancreatobiliary type. Invasive carcinomas derived from intestinal-type IPMNs may be less invasive and slower growing than those derived from the pancreatobiliary type".[73]

The relationship between histological sub-type and overall survival is not as clear cut however. Gastric type has been shown to occur multi-focally but malignant transformation is considered to be rare.[75] However, when invasive adenocarcinoma originates from gastric type IPMN, it seems to be associated with a markedly worse survival compared with those originating from other types of IPMN.[76] Intestinal type IPMN have a higher propensity than other sub-types to progress to invasive carcinoma.[68, 75] Pancreatobiliary type IPMN tends to display aggressive biological behaviour and invasive carcinoma from this sub-type occurs in approximately 50% of cases.[75, 77, 78] usually the oncocytic sub-types of IPMN do not display invasive carcinoma, and if such invasion is present it is usually limited in extent.[79]

Mucin staining has been used in numerous studies to try to differentiate between various 'sub-types' of IPMN.[80-85] Mucins are a family of high molecular weight (>120 KDa), heavily glycosylated proteins produced by epithelial tissues. The commonest mucin proteins (and their loci) which are detected via immunohistochemistry are: MUC 1 (1q22); MUC 2 (11p15); MUC 5AC (11p15); and MUC6 (11p15).

In 2003 an international consensus meeting was held at the Johns Hopkins Hospital in an effort to combine the histological sub-types with the MUC staining, the efforts are summarised in Table 5.[68]

Table 2: Presence or absence of various MUC proteins on IHC by histological sub-type of IPMN.

Histological sub-type	Nuclear Atypia	MUC 1	MUC 2	MUC 5AC	MUC 6
Gastric	Mild/Low-grade	-	-	+	-
Intestinal	Moderate/High-grade	-	+	+	-
Pacreatobiliary	Severe/High-grade	+	-	+	-
Oncocytic	Severe/High-grade	+	-	+	+

The molecular genetics of IPMN have been a focus of research for a number of years. There is general consensus that an activation mutation in KRAS2 is an early step on the path to malignant change in most IPMN.[86] There is less agreement over the role of p53, p16 and SMAD4 mutations.[66] p53 loss of heterozygocity (LOH) has been observed to occur in 38-100% of IPMN with carcinoma (IPMC), p16 LOH is more common (92-100%).[63, 87-89] Loss of SMAD4 expression is significantly less common in IPMN/IPMC than in pancreatic ductal adenocarcinoma.[90, 91] The assessment of expression of p53 has usually been conducted on tissue specimens using immunohistological methods.

The importance of defining subtype can be seen when one considers the likelihood of progression to malignancy. If the clinician can define the probability of invasive change within an IPMN he/she may be better placed to make a management decision based upon the risks versus benefits of resection in a more tailored way for each individual patient.

Once an IPMN is found to have progressed to invasion of the surrounding tissue, this becomes an invasive IPMN. Once again this invasive element has distinct histological sub-types recognised by pathologists: tubular, colloidal, oncocytic.[92] Evidence is growing that these sub-types of invasive IPMN

are associated with differing survival rates – so far several studies have shown that the oncocytic and colloid sub-types compare favourably to 'standard' pancreatic ductal adenocarcinoma, whereas the tubular sub-type is associated with a poorer prognosis.[76, 93-95]

From this summary of the histological descriptions of IPMN it can perhaps be appreciated that the histological reporting of such lesions is cumbersome and far from standardised internationally. It is possible for a single IPMN specimen to be reported as: "side-branch IPMN with intestinal type epithelia (MUC 2 +ve), showing local invasion with oncocytic features". If this were a biopsy of a lesion in the pancreatic head the treatment of choice here would surely be resection. However in the absence of invasion can such an IPMN be safely observed?

Analysis of cyst content to determine malignant potential is a growing area of research. The pancreatic cyst fluid DNA analysis (PANDA) study has so far published results relating to *KRAS* mutations.[62] This study has comprehensively examined the mutations in *KRAS* using direct sequencing. So far in the published literature there have been few reports of attempts at direct sequencing undertaken of p53 in aspirates.[89]

Correlation has already been shown between various DNA/protein markers and malignant potential in cysts. Abe *et.al.* found that there was no over-expression of p53 in 22 'benign' IPMN (when reviewed histologically).[96] As previously mentioned most such studies to date have used immunohistochemistry to identify p53 in tissue.[89]

As mentioned earlier, today over 20% of all pancreatic resections are attributed to IPMNs in high volume surgical centres.[59, 93, 94, 97-99] This raises the question as to why there has been such an increase in the detection of IPMN since its first description in 1982. The answer almost certainly lies in the rise of more advanced and accurate diagnostic modalities such as dual-phase computed tomography (CT) scans and advanced linear endoscopic ultrasound (EUS).[71]

There are currently no formal recommendations for population-based screening of IPMN and the **exact** incidence of pancreatic malignancies occurring within IPMN is unknown.[100] An epidemiological study conducted in California by Le *et.al.* reported 15,000 cases of pancreatic cancer of which 880 (5.9%) arose within cystic lesions.[101] A separate study of IPMN incidence in Korea over 12 years revealed that 1 in 2.4 cystic neoplasms were biopsy proven IPMN.[102]

The difficulties in deciding when to resect IPMN were directly addressed at the conference in Sendai in 2004 which published guidelines for the resection of IPMN.[68] Essentially the consensus opinion was that surgical intervention should be considered for all MD-IPMN and any SB- IPMN which were greater than 3cm in diameter **or** had worrying features on imaging.[68]

As early as 4 years later the Sendai Guidelines were being questioned. In a retrospective study of 190 IPMN which would normally not have been resected under the Sendai criteria, Woo *et.al.* found 53 cases with histological evidence of malignant invasion.[103]

Clearly we have yet to identify a robust method of differentiating potentially malignant IPMN from those which are likely to remain benign. It is also apparent that as imaging techniques become ever more sensitive for small side branch IPMN there will be an increasing burden on the clinical services.

In 2010 the 14th International Association of Pancreatology (IAP) began work on the consensus guidelines for the management of IPMN and MCN which was designed to highlight the shortcoming of the Sendai Criteria as well as offering insights into the accumulated knowledge and understanding of IPMN. These were published in 2012.[104]

1.4 - The European Registry of Hereditary Pancreatic Diseases (EUROPAC)

Founded in 1997 in Liverpool, EUROPAC was conceived by Professor J P Neoptolemos of Liverpool and Professor M Büchler of Heidelberg. Initially the registry existed to collect demographic, symptomatic and genetic data from individuals with hereditary pancreatitis (HP). The Department of Molecular Genetics was located at The Liverpool Women's Hospital and it was here that the early analyses for *PRSS1* mutations were undertaken. After Whitcomb et.al. had identified the *PRSS1* mutation as a causative factor in HP, followed by the description of *SPINK-1* and *CFTR* as potential mechanisms for idiopathic familial pancreatitis, the registry began to amass a large number of kindreds which enabled publication of descriptive papers relating to the incidence of various point mutations.[105-108]

With the description of familial pancreatic cancer (FPC) in 1990 by Lynch et.al. (based upon a case series of familial cases published between 1973-1987) several registries sprung up across Europe and the United States.[109-112] Other registries include: the Seattle Cancer Care Alliance,[113] the American Cancer of the Pancreas Screening Consortium,[114, 115] the German National Case Collection for Familial Pancreatic Cancer (FaPaCa),[116] and the Dutch Registry for Hereditary Tumours.[117]

On the basis that HP and FPC confer an increased risk of pancreatic cancer to individuals within affected kindreds a screening arm of EUROPAC was established in 2007. Prior to this patients on the HP registry already received screening by virtue of the published 40% lifetime risk of pancreatic cancer conferred by HP.[118]

Hereditary Pancreatitis

Inclusion on the HP Registry required pancreatitis occurring in at least two first degree relatives in a single kindred. Those individuals within the kindred who are symptomatic are said to be 'affected' individuals. HP can then be further subdivided as:

"True" HP: the pattern of disease within the kindred is autosomal dominant and/or there is a confirmed mutation of the cationic trypsinogen gene *PRSS1*. The functional affect of this mutation may be to alter the export of trypsinogen, self-activation of trypsin or increased caldecrin activation of trypsinogen.[119] How these mutations cause pancreatic cancer is unclear, but pancreatic cancer is associated with hereditary pancreatitis regardless of the underlying gene mutation.[120]

"Neg All" HP: the pattern of disease within the kindred is autosomal dominant with wild type genotype for *PRSS1* among affected individuals. The presumption being there are further, as yet unidentified mutations, which act in a similar way to *PRSS1* mutations. Copy number variation (CNV) is a type of structural variation, specifically, it is a type of duplication or deletion event that affects a considerable number of base pairs. CNV can be generally categorized into two main groups: short repeats and long repeats. Short repeats include mainly bi-nucleotide repeats and tri-nucleotide repeats. Long repeats include repeats of entire genes.

"Familial Idiopathic Pancreatitis": A non-autosomal dominant cluster of symptomatic individuals within a kindred.

There are geographical differences in the reported penetrance of *PRSS1* mutations, from 40% in Spain, 80% in the USA, 93% in France to 96% in the UK.[121-124]

The risk of pancreatic cancer within these kindreds varies with their genotype. For example an individual with true HP and the p.R122H mutation of *PRSS1* has been shown to have a >50 times increased risk of pancreatic neoplasia.[118] Conversely, an individual with *CFTR* associated familial pancreatitis has a more modestly elevated risk.[125]

The mechanism through which an individual with HP incurs an elevated risk of pancreatic cancer is still contentious – assessment of the functional qualities of the various mutations have yet to provide a clear causative route for the malignant predisposition leading many to think that the changes within the gland as a consequence of chronic pancreatitis increase the risk rather than the mutation *per se*.

Familial Pancreatic Cancer

The term "FPC" applies to families with two or more first-degree relatives with pancreatic cancer that do not fulfil the criteria of any other inherited tumour syndrome.[126]

In the EUROPAC registry an FPC kindred is sub-defined as:

"True" FPC: Pancreatic cancer which has occurred within 2 or more first degree individuals across 2 or more generations (e.g. father and son). Within the EUROPAC cohort there are also kindreds in which there have been more than 2 cases of cancer.

"?" FPC: Kindreds in which there have been 2 or more cases of pancreatic cancer amongst first degree relatives within one generation (e.g. brother and sister).

"Other" FPC: This includes kindreds in which there have been at least one case of pancreatic cancer in conjunction with either; an associated mutation known to predispose to pancreatic cancer, or more than two cases of another cancer known to predispose to pancreatic cancer in the absence of any proven mutation.

When discussing FPC it is important to exclude families that fit within other cancer syndromes or defined syndromes predisposing to conditions such pancreatitis, that are associated with high risk of pancreatic cancer. Segregation analysis of FPC families suggests that there is a rare major gene conferring predisposition.[127] Other studies claim an autosomal dominant transmission.[128] The question of autosomal dominance has been the subject of some debate, but as cancer is widely accepted to develop by multiple mutations ("hits") any disease allele that can predispose to cancer when homozygous must also predispose to cancer in a heterozygous individual as a result of the potential somatic loss of the wild-type allele. Therefore, the issue is not whether inheritance is autosomal dominant, but rather the age of penetrance. EUROPAC, along with its German sister registry FaPaCa, was instrumental in demonstrating the phenomenon of 'anticipation' (the lowering of age of onset from earlier to later generations) among individuals of FPC kindreds.[129, 130]

One large American FPC kindred (known as Family X) did demonstrate autosomal dominant inheritance of pancreatic cancer with high penetrance, and anticipation among younger generations.[131] Subsequent linkage analysis pointed to the *PALLD* gene (4q32-34) which encodes palladin and the germline mutation p.239S was identified as the oncogenic trigger in these individuals.[132] Unfortunately subsequent analyses of families by the various National cancer registries failed to demonstrate this mutation elsewhere.

In the United States the National Familial Pancreas Tumour Registry (NFPTR) estimated the relative risk and cumulative lifetime risk of the development of pancreatic cancer in FPC kindreds to be 6.4% and 12%, respectively for individuals with two affected first degree relatives, and 32% and 16-30% for individuals with three affected first degree relatives, respectively.[133, 134] It should be noted, however, that within the NFPTR some groups include kindreds with three or more cases of pancreatic cancer in non-first degree relatives as 'FPC kindreds'.[135, 136]

Screening

A quantitative analysis of the timing of the genetic evolution of **sporadic** pancreatic cancer indicated a time span of at least 10 years between the occurrence of the cancer-initiating mutation and the birth of the 'founder' malignant cell.[134, 137] This creates an ideal window for screening to detect malignant transformation and affect potentially curative therapy before the disease progresses to become incurable. The optimal method of screening for early cancer in individuals from FPC and HP families is still being developed.[113-117, 126, 138-140]

Although there is a potential window of opportunity for the detection of pancreatic intraepithelial neoplasia (PanINs), once malignant transformation occurs then metastases develop very early, necessitating the need for pre-symptomatic screening.[33, 139-141] A number of screening programmes are reliant on imaging of early tumours using endoscopic ultrasound (EUS) including the Seattle Cancer Care Alliance,[113] the American Cancer of the Pancreas Screening Consortium,[114, 115] the German National Case Collection for Familial Pancreatic Cancer (FaPaCa),[116] and the Dutch Registry for

Hereditary Tumours.[117] The screening approach undertaken by EUROPAC has been to identify molecular alterations in pancreatic juice predictive of cancer in parallel with imaging evidence of neoplasia.[139, 140] This has the potential advantage of a longer lead time in diagnosis thereby not only increasing the interval between screening periods but also minimising the risk of early metastases.

There has been a direct comparison of different imaging techniques carried out within a screening study; EUS showed a greater sensitivity for detecting pancreatic lesions in asymptomatic individuals than CT or MRI.[114] Compared to CT, EUS has a superior rate of detection of pancreatic tumours, 94-100% vs. 69-85%.[142] Perhaps more importantly in the context of screening asymptomatic individuals, CT comes with the considerable disadvantage that it involves a dose of radiation equivalent to 10-20mSv/1-2 rem (500 – 1,000 chest x-rays).[143] This is of particular concern as it is likely some of the screening participants will have DNA repair defects.[144-146] Therefore EUS is currently the imaging modality most extensively used in screening.[113, 140, 147-149]

The results and interpretations of the different screening studies have been variable; cancers have been missed and the cost/benefit ratio has been questioned, [117] yet some groups have reported exceptionally high diagnostic yield.[116, 117, 150] Certainly there is a need for improved screening modalities that are safer, cheaper and more effective. A serum marker would be ideal as it is would be minimally invasive. However, the best of these currently, CA19-9, lacks specificity and as there is no expression of this marker in 5-10% of individuals who are Lewis^{a-b-}, it also lacks sensitivity. This has led the American Society of Clinical Oncology to reject it for screening.[151, 152]

Samples of pancreatic juice obtained from the pancreatic duct at endoscopic retrograde cholangiopancreatography (ERCP) have been used for molecular analysis as part of the EUROPAC screening programme since 1998. The molecular analysis is an adjunct to imaging in an effort to determine the optimal frequency of EUS. Pancreatic juice is the secretion most intimately in contact with tumours and so may contain either tumour cells sloughed from the duct or cellular components including DNA liberated from cancer cells by necrosis.[153]

In Chapter 5 I will present the screening outcomes from patients who were recruited to the registry and screened prior to and during my period of research (2010-2014).

1.5 - p53

Wild Type

Initially thought to be an oncogene, the p53 protein of the *TP53* gene was first described in 1979 and published in *Nature* in 1984.[154] p53 is now recognised as a transcription factor which is found to be present in about half of all malignant tumours regardless of their cell of origin, a single mutant allele of which leads to Li Fraumeni Syndrome.[155, 156]

The mechanisms by which p53 accomplishes all of its biological functions are not completely understood, it has been shown that p53 specifically binds to sequences of DNA within the intron or promoter sequences of genes which are known as p53 responsive elements (p53RE).[157-160] Through various mediators and transcriptional activity p53 is able to inhibit cell proliferation or induce apoptosis and is therefore an active molecule in neoplasia especially where dysplasia is present.[161]

The most described activity of p53 is probably apoptosis in response to various stimuli; it was only in 2009 that a comprehensive mechanism of increased mitochondrial outer-membrane permeability was shown to be the means by which this was achieved.[162, 163]

p53 can thus be described either with reference to the triggers for activation, e.g. DNA damage, oxidative stress, oncogene activation, hypoxia; or by its effects as described above. It is also known, however, that p53 does more than simply affect the cell cycle – numerous down-stream effects have been and are being documented.[164] Using various animal models p53 has also been implicated in the development of the neural tube in embryos with p53 null mice developing significant neural tube defects.[165, 166] Even earlier than the embryonic stage, p53 is known to increase fertility by enhancing the chances of implantation of a fertilised egg;[167, 168] even the skin tone and propensity to a sun-tan may also be determined, in part, by the same gene.[168, 169]

It is important to note that the effects of p53 are not limited to cancer – work has shown that as we age we garner more p53 mutations which in turn lead to increasing cellular signs of aging and

conversely accumulated wild-type p53 is associated with increasing signs of aging in murine subjects.[170, 171]

As well as regulatory physiological roles, p53 is also active in certain diseases and conditions which are non-malignant – it promotes a senescence response to insulin in adipose tissues leading to diabetes as well as being shown to promote apoptosis in response to ischaemia in both neurons (stroke) and cardiac muscle (myocardial infarct).[172-174] As well as stimulating apoptosis there has been evidence to suggest that by enforcing it's role of 'cellular guardian' p53 mediated destruction accelerates neurodegenerative conditions such as Alzheimer's, Parkinson's and Huntington's.[175-178]

p53 is, to a certain extent, auto regulated via Mouse double minute 2 homolog (MDM2) which is an E3 ubiquitin-protein ligase.[179] MDM2 recognises the N-terminal of activated p53 and is an important step in the ubiquination pathway (in combination with a E2 ubiquitin-protein ligase) which eventually leads to degradation of p53 via the proteasome activation.[180] MDM2, produced in tandem with p53 has also been shown to be an inhibitor of p53 post translational activity – acting as a negative feedback loop.[181-183]

At its most basic level p53 encodes a 393-amino acid protein which will bind with DNA.[184, 185] When activated p53 will bind with DNA and stimulate the production of many transcripts including miR-34a and WAF1/CIP1 also known as p21.[186, 187] When p21 combines with CDK2/cyclin the cell cycle is halted at the G1/S checkpoint, thus rendering the cell amenable to various DNA repair mechanisms before progressing to S phase.[157, 188, 189] When p53 binds to CDK1/cyclin B it halts the cell cycle at G2/M. As p53 responds to DNA damage, wild-type p53 can therefore be described as the "guardian of the genome".[190]

Cells lacking normal p53 suppressor gene activity, such as most neoplastic cells, suffer from genomic instability resulting from loss of checkpoint activity during the G1 phase, eventually culminating in gene amplification, aneuploidy and other chromosomal aberrations. These abnormalities then contribute to the clonal evolution of cancer cells and tumour progression.[191, 192]

p53 activation is also triggered by the presence of free oxygen radicals which can be produced as a consequence of chronic inflammation such as that seen in chronic pancreatitis or as a consequence of ductal obstruction in pancreatic cancer.[182, 193]

Hypoxia, too, is a potent stimulator of p53 – this mechanism is most strikingly demonstrated as a consequence of expansive cellular proliferation with insufficient angiogenesis as is seen in malignancy.[182, 194, 195] Interestingly various mutant genotypes of p53 have been identified which are resistant to hypoxia-induced apoptosis, such variants are common in larger tumours.[196, 197]

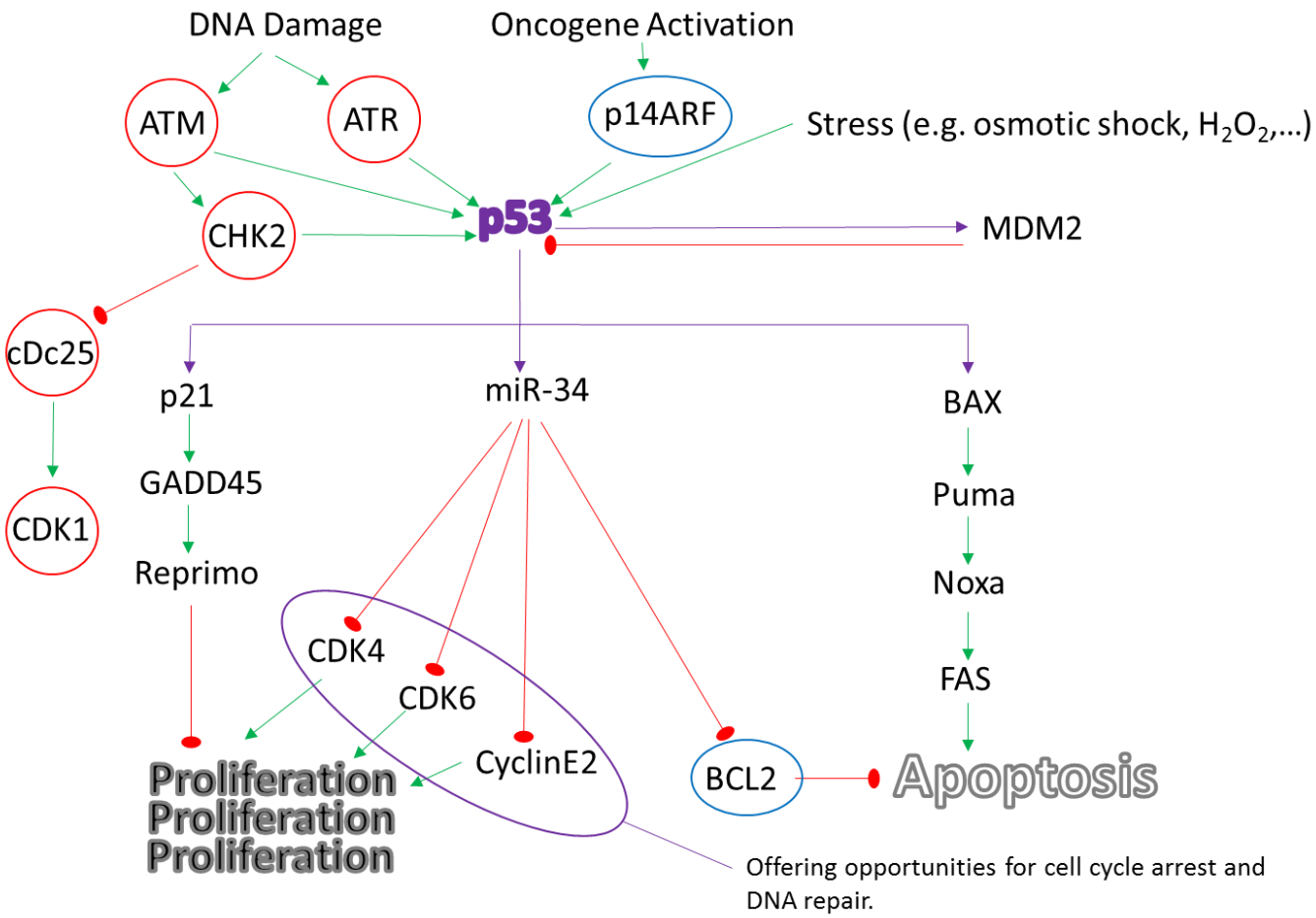


Figure 1: Select p53 pathways (after Brady & Attardi).[198]

Prevention of autophagy may also be among the possible consequences of p53 activation in response to established tumoreogensis, this allows a cell to catabolise macromolecules for reuse, either as a survival strategy under stress conditions or as a means to remove harmful, damaged structures.[199] It has been shown that even minute levels of wild-type p53 present within the cytoplasm can impede

the formation of autophagosomes thus effectively blocking this mechanism as a route for evading detection and continued survival of malignant cells.[199, 200]

Mutant

A p53 mutation is one of the most frequently found mutations in human cancers.[181, 201] As already discussed a mutation in p53 may lead to p53-dependent apoptosis suppression among pre-malignant lesions and subsequently provides selective pressure for further p53 suppression.[202, 203] Of course suppression of p53 activity can occur either through overexpression of MDM2, a mutation within p53 itself or mutations downstream among p53 pathways.[194] In murine models p53 null mice get completely penetrant cancer phenotypes.[204]

It is estimated that 50% of all human cancers harbour a mutation in p53 – the incidence of this mutation, and indeed the timing of the mutation within the neoplasia-malignancy progression varies depending upon the tissue type.[181, 182, 205, 206] Because of its inherent tumour suppressor activity, inactivation of p53 without mutation can also lead to spontaneous tumorigenesis.[207] It is interesting to note that one theory of the evolution of p53 suggests that as organisms have evolved to live longer p53 has become more relied upon for neoplasia detection – given the significance of its role in physiological processes and pathological conditions it is thought probable that this was not the original role of p53 in simple organisms.[208]

Because p53 functions by forming a tetramer of 4 individual p53 protein molecules, a single mono-allelic mutation can exert a dominant negative effect in the presence of a majority of wild type expressed protein by disrupting the formation of an effective tetrameric transcription factor.[209] The presence of mutant p53, rather than the lack of wild type, may confer a survival advantage for the evolution of tumour cells.[210] This has been postulated as the “gain of function” hypothesis for mutant p53.[211, 212] Some mutations can be shown to prevent only the cell cycle arrest function of p53 and others only the apoptotic function, of course a combination of such mutations would harbour significant malignant potential.[213-215] Accordingly, human tumours with mutant p53 are associated

with poor patient prognosis and drug resistance.[205, 216] More pertinent, perhaps, is the knowledge that the side effects of chemotherapy and radiotherapy are to a large extent mediated by p53.[217]

p53 is also known to repress the action of other genes by directly binding or occluding the binding sites for other transcription factors.[218, 219]

The relative effect of such mutations can be measured by monitoring the expression of auto-regulatory proteins (e.g. MDM2) and downstream markers (e.g. p21, BCL2). A description of the function of each known mutation of p53 (there are over 8000) can be found at the online International Agency for Research on Cancer (IARC) p53 database (http://p53.iarc.fr/*TP53*GeneVariations.aspx); along with the incidence and effects of the mutation using these surrogate markers. As early as 1994 it was shown that p53 mediated apoptosis can occur even in the presence of inhibitors of p53 DNA binding, thus it has been shown that there is cytoplasmic activity of p53.[220]

The vast majority of p53 mutations which occur in cancer cause an inability of p53 to bind to sequence specific regions of genes and thus disables the regulatory mechanism, it has been shown that p53 mutations typically occur within the DNA-binding region and involve either DNA contact residues or residues important for conformational structure, both resulting in loss of DNA binding.[221, 222] More than 80% of p53 mutations occur as a single amino-acid substitution resulting in the synthesis of a full length-stable protein which lacks DNA binding specificity, rather than the usual frame-shift or nonsense mutations found in other tumour suppressor genes.[181, 223]

As a consequence of the action of p53 the majority of described deleterious mutations occur within the DNA binding region of p53. Exons 5, 6, 7 and 8 have been shown to contain over 90% of the

described mutations so far, 28% of these have been reported to occur at one of 6 codons (p.R175, p.G245, p.R248, p.R249, p.R273, p.R282).[224]

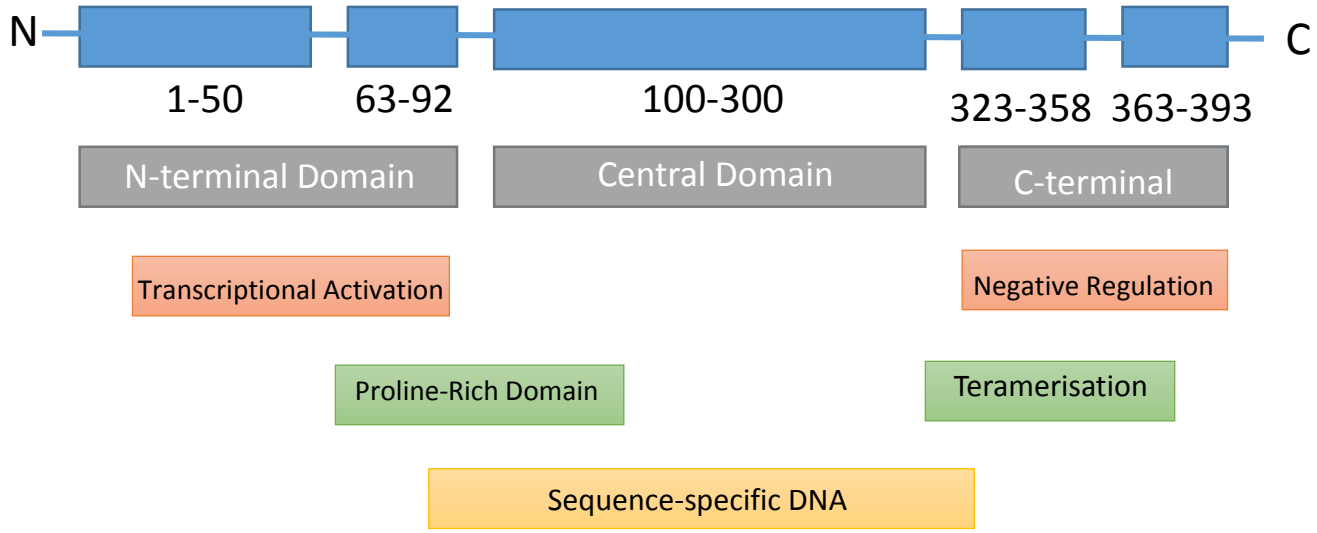


Figure 2: p53 protein structure.

P53 and the pancreas

PDAC is one type of cancer in which p53 has been shown to significantly impact disease progression. The most popular model for PDAC development is that it usually arises from a background of PanIN which can persist indolently for years, mutation of p53 within such tissue, however, is associated with invasive metastatic PDAC in 75% of cases.[225] p53 is a recognised late stage mutation in PanIN and has also been shown to be a contributing factor in local recurrence following presumptively curative resection.[226-229] Further evidence of the metastatic role of p53 in PDAC can be found in the conclusion of Morton, *et.al.* who demonstrated that, in a murine model, PDAC driven by oncogenic *Kras* was unlikely to metastasise in mice with wild type p53 compared to those with concomitant mutant p53.[230] Recent work has identified platelet-derived growth factor receptor b (PDGFRb) as a mediator of the effects of mutant p53 on invasion and metastasis in both a murine model and human PDAC cells.[231]

In cystic lesions of the pancreas, such as IPMN, the role of mutant p53 is less well described. Mutations in p53 are thought to be less common in cystic lesions compared with solid PanIN derived precursor lesions, and in IPMN specifically the correlation appears to be with the grade of dysplasia rather than the epithelial subtype.[87, 232, 233] One study estimated that the incidence of p53 mutations in IPMN were

50% lower than in PanIN.[234] A recent next generation sequencing project of various cancer associated genes within IPMN found that only 10% carried mutant p53 (n=5), all of these mutations were found within high grade IPMN and 3 of them within codons 5 or 6.[89] Until recently, the assessment of p53 expression was usually made using IHC rather than direct sequencing. As a result it is difficult to perform a systematic review of p53 mutation expression among cystic lesions of the pancreas. Even with IHC, however, aberrant p53 expression was still only identified amongst IPMN with carcinoma as opposed to those with dysplasia (5/13 vs. 0/16).[89, 90]

In terms of prognostic markers, the Johns Hopkins Group have reliably and recurrently identified aberrant CpG island hypermethylation, GNAS mutations and NRAS mutations as drivers of malignant change in IPMN.[89, 235, 236] p53 has been less commonly seen in these genetic profiling experiments, this may be because the late development of p53 renders the malignant IPMN unresectable by virtue of local spread and thus tissue is not available or perhaps, in contrast to PanIN, p53 is not a common mutation in malignant transformation of IPMN.

Chapter 2
AIM & OBJECTIVES

2 – Aim & Objectives

Identify a novel genetic marker which can predict malignant transformation or overall survival in individuals with lesions which are high risk for developing pancreatic malignancy. This marker should, ideally, be suitable for use in an appropriate population.

1.) Identify suitable candidates for screening

2.) Identify the number of lesions seen in a pilot study

3.) Identify the chance of IPMN developing into pancreatic ductal adenocarcinoma (PDAC)

4.) Identify means of improving yield of genuine positives in screening

Chapter 3
HYPOTHESIS

3 – Hypothesis

That there will be biomarkers that can adequately stratify screened individuals to allow secondary screening for pancreatic cancer to be effective.

Chapter 4
METHODS

4.1 - Samples Obtained

In line with the stated aims of my thesis I needed to obtain samples of resected pancreata which contained cystic neoplasms. It was also important that these samples had matched accurate survival and follow-up data.

Tissue samples from this project were donated by Professor Markus Büchler on behalf of Heidelberg University Hospital, Heidelberg, Germany.

I chose to approach the Pancreas Research Group in Heidelberg (on the advice of my supervisors) for the following reasons:

1.) The group resect far more cystic lesions of the pancreas than any centre in the UK. This is mainly due to the differences in management (and the confines of the National Health Service in the UK) of such lesions. In Germany, suspicious lesions are routinely resected whereas surveillance imaging is normally undertaken in the UK if there are no overt features of malignancy. [58, 95, 101, 237]
2.) Obtaining tissue samples from outside of my research group would make it much easier to stay blinded to the histological and survival data of the patients.
3.) Our group already had strong collaborative ties to the Heidelberg Pancreatic Unit through work published as part of the various ESPAC trials. [46, 238-243]

73 separate unique snap frozen tissue specimens were transported to the NIHR Pancreas Biomedical Research Unit (PBRU) in dry ice at -80°C and were received in June 2012. Each was randomly allocated an alphanumerical identifier in the format CYSTXXX (where XXX represented sequentially increasing numbers). These new 'Liverpool' identifiers and their corresponding locations within the -150°C freezer NIHR PBRU tissue bank were recorded on the Laboratory Information and Management System (LIMS) in accordance with Good Clinical Practice (GCP).

As already outlined, my fellow researchers working on this project and I were blinded to any demographic, histological or survival data for the patients from whom these samples had been obtained.

The samples now labelled CYST001 to CYST072 were then individually sliced into three separate pieces of roughly equal size without any reference to the presence or absence of macroscopically apparent tumour, duct, or cyst within them. This dissection was not performed by a pathologist and was undertaken using separate scalpel blades on a non-porous surface cleaned with 70% ethanol between samples. These 'sub-divisions' of the original samples were labelled as CYST001a, b or c. One third of each sample (a) was destined to be used for immunohistochemistry (IHC) as part of this project; one third (b) for DNA analysis; the final third (c) was returned to storage at -150°C in anticipation of further work or validation related to any findings of this project.

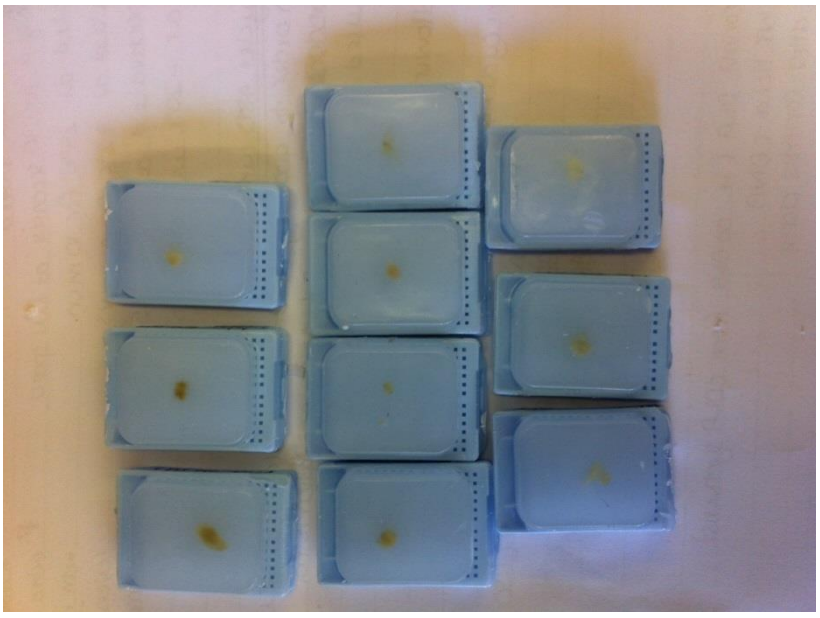


Figure 3: Tissue blocks created from Heidelberg samples ready for slicing with the microtome and then IHC.

The samples which were to be used for IHC (a) were transferred to the Royal Liverpool University Hospital Department of Pathology for processing in 10% formalin and were each embedded into a separate paraffin block, labelled and stored in a designated tissue storage cabinet at room temperature and recorded on the laboratory information and management system (LIMS).

4.2 - Overview of Immunohistochemistry

As previously detailed, the tissue samples which were destined to be used for IHC analysis had been prepared using formalin fixation and paraffin embedding. This ensured ease of tissue slicing when preparing slides for pathological review and IHC.

Prior to consideration of IHC I decided that it would be sensible to ensure that each specimen had exposed epithelial cells to bind with my chosen antibodies. In this vein I enlisted the assistance of Professor Fiona Campbell, Consultant Histopathologist at the Royal Liverpool University Hospital. Once I had prepared the samples by slicing on a microtome (4µm) and mounting onto microscope slides I performed standard haematoxylin & eosin (H&E) staining and presented them to Professor Campbell for review.

The first such review took place on 9th January 2013 and of the 72 slides presented 21 (29%) were identified as cystic lesions with sufficient epithelial cells to warrant IHC. Over the following 2 months repeated 4µm slices were taken of the remaining 52 samples, stained with H&E and reviewed by Professor Campbell. I was aware that as I had sliced the original frozen tissue samples with no reference to the gross pathology it was possible that the cystic neoplasm originally resected in Heidelberg may not actually be present in these slices. To combat this I ensured 10 4µm slices were removed and stored prior to each new H&E stain, this way if I did start to cut into the cystic neoplasm I could ask for the previous slices to be examined. Eventually after three 'rounds' of histological review I was left with 22 (30%) samples which were confirmed as displaying epithelial cells consistent with a cystic neoplasm and which were thus suitable for IHC analysis.

As discussed in 1.3 - Cystic Lesions I had chosen to assess these 22 tissue samples for expression of the proteins MUC1, MUC2, MUC5AC and MUC6. In preparation, I mounted 10 4µm slices of each tissue sample onto separate microscope slides and allowed them to dry in an incubator at 37°C for 48 hours. I determined that it would be fruitless and costly in terms of both time and money to try and

IHC stain those remaining 50 samples which had been assessed as not displaying epithelium as there would be no specific antibody binding and so results would be meaningless. As a positive control and quality assurance measure I was also provided with 4 tissue blocks of cystic neoplasms which had been resected at the Royal Liverpool University Hospital and which had already undergone IHC staining with MUC1, 2, 5AC and 6 as part of their clinical pathological assessment. This staining had taken place within the Department of Pathology in the Clinical Pathology Association (CPA) accredited laboratory and so their findings could be considered as a gold standard against which I could measure my staining outcomes.

MUC Antibodies

All of the MUC antibodies were ordered from Leica (Leica Biosystems, Newcastle, UK) and arrived anhydrous. All were mouse monoclonal antibodies, their catalogue numbers are given over the following pages. They were each reconstituted with the required volume of molecular grade DNA free sterile water as indicated on their respective datasheets. After this each antibody was stored at 4°C in a laboratory refrigerator.

Control Tissue Samples

The suggested positive and negative control tissues for each antibody was as detailed in their datasheet (Leica Biosystems, Newcastle, UK). These tissues were obtained with thanks from the Liverpool Tissue Bank.

Tris-EDTA Buffer pH9.0

I created a 10x stock solution of Tris-EDTA buffer, using the following formula:

- 12.1g of tris(hydroxymethyl)aminomethane (Tris)
- 3.7g of Ethylenediaminetetraacetic acid (EDTA)
- 800mL of distilled water

This was brought to pH of 9.0 using pellets of NaOH and diluted to 1000mL using distilled water. This stock was then stored at 4°C in a laboratory refrigerator.

TBS

I created a 20x stock solution of Tris Buffered Saline (TBS), under the guidance of Mrs Elizabeth Garner, using the following formula:

- 121.4g of Tris
- 175.32g of NaCl
- 800mL of distilled water

This was brought to pH of 7.6 using aqueous HCl and diluted to 1000mL using distilled water. This stock was then stored at room temperature.

Scott's Tap Water

- 30g of Magnesium Sulphate ($MgSO_4$)
- 2g of Sodium Bicarbonate ($NaCO_3$) 2.0 gm
- 3L of distilled water

Acid Water

- 10mL of 10M HCl
- 900mL of distilled water

4.2.1 - Immunohistochemistry Methods

Each of the antibodies arrived with a specific datasheet which outlined the manufacturers' suggested protocol for optimal results. Optimisation of these antibodies took a considerable length of time and I am indebted to Mrs Elizabeth Garner who made the breakthrough suggestion that I should incubate MUC 2 overnight rather than the 'suggested' 1 hour.

In essence there are three variables which should be considered when performing IHC:

1) Antigen retrieval
2) Primary antibody concentration
3) Incubation Time

I set about testing each of these in turn to try to optimise the conditions. After each IHC run I presented the samples to Professor Campbell for assessment of which slides had optimal antibody uptake.

Antigen retrieval

The datasheets for each of the antibodies recommended "high temperature antigen retrieval using 0.01M citrate retrieval solution at pH6.0". I therefore began my IHC experiments with a single run of 8 slides (one positive and one negative control tissue slice for each antibody) and a constant primary antibody concentration of 1:50 dilution. The incubation time was similarly standardised at 60 minutes for the primary and 60 minutes for the secondary antibody.

Some 4 hours later none of the positive (nor indeed negative) slides showed any antibody uptake. I reasoned that the concentration must be sufficient (it being the minimum suggested for each antibody) and that 60 minutes incubation was standard according to the data sheets. After reviewing the literature specifically related to MUC antibodies in pancreatic lesions it became apparent that an EDTA based buffer may be more appropriate. I repeated the experiment with EDTA Buffer (pH6.0)

which yielded some weak staining for MUC 1, 5AC and 6 but no staining for MUC 2. Finally another run was conducted with EDTA at pH9.0 and I also increased the concentration of MUC 2 antibody to 1:10 whilst maintaining the others at 1:50. There was now very strong staining (with background cells positive) in MUC 1, 5AC and 6 but still nothing in MUC 2. I concluded that EDTA buffer at pH9.0 was the optimum method of antigen retrieval for the remaining antibodies (excluding MUC 2).

Primary Antibody Concentration

After optimising retrieval, IHC analysis was repeated on the positive control tissues using concentrations of primary antibody at: 1:100; 1:200; 1:500 and 1:1000 to find the optimum stain of epithelium without excessive background artefact.

After review by Professor Campbell I found that the optimum concentrations were:

- MUC 1 = 1:200
- MUC 5AC = 1:100
- MUC 6 = 1:200

Incubation Time

Incubation time was established at 60 minutes for all other antibodies but was increased to 12 hours at 4°C for MUC 2. I maintained the retrieval methods already established (high temperature EDTA pH9.0) and performed a run at concentrations of 1:50; 1:100; 1:200 and 1:1000 leaving the primary antibody on for 12 hours in the cold store (4°C) and the secondary antibody on for 60 minutes at room temperature. Finally, the positive tissues stained appropriately and the optimum concentration was identified as 1:100.

For each run of IHC I focussed on a single antibody and used a maximum of 10 sample slides in conjunction with one positive and one control sample. Thus each antibody was run three times (26 samples).

Assessment of IHC

Each slide was reviewed by Professor Campbell who reported whether the tissue staining was positive or negative.

4.2.2 – Materials for MUC Staining

MUC 1 Specification

Novocastra™ Lyophilized Mouse Monoclonal Antibody Muc-1 Glycoprotein.

NCL-MUC-1 (Leica Microsystems, Newcastle, UK)

Control Tissues

Positive: Normal Pancreas

Negative: Skeletal Muscle

MUC 2 Specification

Novocastra™ Lyophilized Mouse Monoclonal Antibody Muc-2 Glycoprotein.

NCL-MUC-2 (Leica Microsystems, Newcastle, UK)

Control Tissues

Positive: Duodenum

Negative: Skeletal Muscle

MUC 5AC Specification

Novocastra™ Lyophilized Mouse Monoclonal Antibody Muc-5AC Glycoprotein.

NCL-MUC-5AC (Leica Microsystems, Newcastle, UK)

Control Tissues

Positive: Stomach

Negative: Tonsil

MUC 6 Specification

Novocastra™ Lyophilized Mouse Monoclonal Antibody Muc-2 Glycoprotein.

NL-MUC-6 (Leica Microsystems, Newcastle, UK)

Control Tissues

Positive: Duodenum

Negative: Tonsil

Preparation of Paraffin Embedded Tissue

Firstly the tissue was placed in xylene for 5 minutes followed by another agitation in fresh xylene for a further 5 minutes. This was repeated for a third time. The tissue was then washed in 3 separate containers of fresh 100% ethanol for 3 minutes each with gentle agitation. The tissue was next placed in reducing concentrations of alcohol (95%, 75%, 30%) for 2 minutes each with gentle agitation. Finally the tissue was left to 'rest' in a container of distilled water for 5 minutes.

Antigen Retrieval

To retrieve the antigen I created 2L of EDTA Buffer pH9.0 by combining 200mL of stock with 1,800mL of distilled water and poured this into a pressure cooker which was brought to the boil. The slides were placed in the solution in a metal rack and the pressure cooker was sealed and set to 1 bar (14.50 psi, 99.9 kPa). Once the pressure was reached the slides were left to boil for 2 minutes and 30 seconds.

Once the time had elapsed the pressure was released and the pan was immersed in a sink full of cold water. Once the rack had cooled it was removed and placed into a container of distilled water.

One Litre of TBST was created by combining 50mL of stock TBS (p.42) with 949.5mL of distilled water and 0.5mL of Tween (Polysorbate 20), this was placed into wash bottle.

Excess water was gently removed from each slide with a clean piece of tissue and a continuous circle was drawn around the tissue with a PAP-pen (Sigma-Aldrich, St Louis, MO, USA) which creates a hydrophobic barrier when dry. Each specimen was covered with sufficient drops of Endogenous Peroxidase Block (Dako, Denmark) to form a raised 'bubble' within the hydrophobic barrier. These were then incubated at room temperature for 10 minutes. Each slide was next irrigated with TBST sufficient to remove the Endogenous Peroxidase Block and leave each sample covered with TBST for 5 minutes. The washing of the slides was repeated 4 further times.

Primary Antibody

120µL of a 1:200 dilution of MUC 1, 1:100 MUC 2, 1:100 MUC 5AC and 1:200 MUC 6 was placed onto each slide, covering each tissue specimen, this was then incubated under a cover for 60 minutes at room temperature. After 60 minutes each slide was irrigated with TBST sufficient to remove the primary antibody and each sample was covered with TBST for 5 minutes. This process was repeated a further 4 times.

Secondary Antibody

Each specimen was covered with 120µL of Labelled Anti-Mouse Antibody (Dako), and incubated under a cover for 60 minutes at room temperature. In the case of MUC 2 the slides were left for 12 hours at 4°C. The slides were then irrigated with TBST sufficient to remove the Anti-Mouse Antibody and left covered with TBST for 5 minutes, this was repeated a further 4 times.

Antibody Staining

Firstly 5 drops of Chromagen Substrate Buffer (Dako, Denmark) were combined with 5mL of distilled water and 120µL was placed onto each slide, covering each tissue specimen. Secondly the slides were incubated under a cover for 10 minutes at room temperature. The slides were then individually

irrigated with TBST sufficient to remove the Chromagen Substrate Buffer and left covered with TBST for 5 minutes. This process was repeated a further 4 times before the slides were immersed in distilled water for 5 minutes.

Preparation of Tissue for Assessment

Each slide was placed into haematoxylin for 1 minute then rinsed under running tap water for 1 minute until water ran clear. The slides were then placed into Acid Water (p.43) for 1 minute and rinsed again for a further 1 minute. Next, the slides were immersed in Scott's Tap Water (p.43) for 1 minute and rinsed thereafter.

Following this the slides were once again immersed in ethanol of increasing concentration for 1 minute each – 30%, 75%, 95%, 100%, with gentle agitation before being placed in three separate containers of xylene for 1 minute each.

Finally, 1 drop of DPX mounting glue (Sigma-Aldrich, St Louis, MO, USA) was placed over the tissue which was then covered with a microscope cover slip. The tissue was allowed to dry at room temperature for 48 hours before viewing under microscope.

4.3 – Overview of Mutational Analyses

The Personal Genome Machine originally manufactured by Ion Torrent and now by Thermo Fisher will be described here, in line with common usage, as the Ion Torrent next generation sequencer. It is capable of generating a sequence of DNA by flooding a well, which contains the reference sequence, with alternating nucleotides (A, C, G or T). If the exposed base of the reference sequence is Adenine then when the well is flooded with the complimentary base Tyrosine there will be a polymerase reaction between the nucleotide and the terminal ribose of the nascent strand and as a consequence a H^+ ion will be released. At the base of the well a pH meter will recognise the H^+ ion and will record that a reaction has taken place. The processor will be aware that the base released was T and so will report the length of DNA being sequenced as containing an A. If there are two nucleotides then twice as many protons would be released and the pH meter would record accordingly. Should the base which is used to flood the well be non-complimentary there will be no polymerisation, no H^+ release and so no change in pH detected. Each well is flooded with a given base every 15 seconds until the end of the template fragment is reached.

The well is contained within a small microchip, the number of wells available on a chip is denoted by the reference number: 314 has 4million wells, 316 has 6million etc… Clearly if 100% of wells are filled with the same sequence of reference DNA then 4 million sequences, known as reads, would be produced. Alternatively 4 million separate reference sequences of DNA could be placed upon a chip which (again assuming 100% coverage) would result in a unique read per well. Of course one can never guarantee that every well will receive a strand of reference DNA. A refinement to this approach is to use more than one sample (patient) and for this it is necessary to add a unique sequence of bases which enable identification of each sequence. In relation to this point the ideal reaction would entail an equal distribution of individual samples across the wells.

Prior to the reference sample(s) of DNA being added to the chip they must be enriched and impurities (e.g. primer-dimers) removed as far as is practicable to ensure the quality of the reads produced.

It can be seen that there is an optimum equilibrium between the number of samples placed upon a chip and the resulting quality of the sequences produced. In part this depends upon the 'depth' of the sequences produced. This is defined as the number of bases called at a given locus, the greater the number of reads the 'deeper' the read and the more confidence can be inferred that the base called is an accurate facsimile of the reference sequence.

I have already alluded to the fact that the optimum confidence in the accuracy of sequence output would come from the maximum number of wells being filled solely with that reference sequence. However, the cost of analysis of a single chip on the ion torrent (including necessary reagents) would be approximately £400. To analyse all of my samples (n=42) on individual chips and perform the required 10 repetitions would therefore cost 10 x 42 x £400 = £168,000.

Another option would be to run a single chip and design 420 individual primers for each of the 16 fragments of *TP53* to be analysed. The mean primer length including ion torrent adaptors is 42 mer. The number of additional bases required to create 420 unique sequences would be 5 per primer (4^5). The resulting mean length would be 47 mer per primer at a cost of £0.20 per base this would equate to 420 x 16 x 47 x £0.20 = £63,168 (+£400 for the chip). Of course this would only give a single read per sample at best – assuming 100% of the wells were filled. Confidence would be correspondingly poor.

Using a combination of: average depth required (5,000 reads); number of samples to be processed (42); and the cost of chips and primers I calculated that the optimum financial ratio was to have three patients (each with 10 repetitions) per chip. This would necessitate 480 primers with three base barcodes, thus 45 mer. Total cost 480 x 45 x £0.20 = £4,320. I would also need 14 chips (42/3) and sufficient reagents at a cost of £5,600, giving a total of £9,920.

If there was 100% coverage of the chip I could expect 5,000 reads at each locus of every fragment analysed. With 10 barcodes per sample I could hope to achieve sufficient confidence that the sequences produced were accurate.

4.3.1 - DNA Extraction

The first step in the analysis of DNA from the frozen samples was to extract the nucleic acid from the tissue. This was performed using the following protocol, modified from Qiagen DNeasy Blood & Tissue Kit (Qiagen, Germany). Forty-eight hours prior to the commencement of DNA extraction the tissue samples labelled CYST001b to CYST072b were removed from -150°C storage to -20°C and recorded on LIMS.

The principle behind DNA extraction is that cells need to be lysed and the released DNA purified, bound to a matrix, washed to remove RNA and impurities and then eluted from the matrix into a suitable elution buffer (usually molecular grade DNA free sterile water).

Lysis

A pestle, mortar and metal forceps were 'sterilised' with 90% ethanol and then left under UV light for 30 minutes, the mortar was then transferred to a suitable container full of ice and placed on a balance. Ten milligrams of frozen tissue from one sample (e.g. CYST001) was placed into the mortar using the sterile forceps whilst wearing sterile gloves. The mortar was then removed from the balance but left inside the ice box to allow the tissue to be macerated using a single use, sterile, size 15 scalpel blade (Swann-Morton, England). The tissue was then crushed using the pestle.

This crushed tissue was next transferred into a sterile 1.5mL Eppendorf tube using the blade and forceps and 180µL of Buffer ATL (Qiagen, Germany) was added, as was 20µL of Proteinase-K (Qiagen, Germany). The Eppendorf was then placed into a heat block at 56°C for 4 hours and vortexed 3 times each hour. Finally the Eppendorf was centrifuged at maximum speed for 15 seconds (RCF 60,000).

Purification

A further 4µL of RNAse A (Qiagen, Germany) was added and the sample vortexed for 2 minutes. Following this the Eppendorf was centrifuged at maximum speed (RCF 60,000) for 15 seconds before another 200µL of Buffer AL (Qiagen, Germany) was added. The sample was once again subjected to a 15sec vortex.

Binding

The samples were placed into a heat block at 70°C for 10 minutes before being spun at 60,000 RCF for 15 seconds.

Washing

To wash the samples, 200µL of 98% Ethanol was added prior to a 15 second vortex and 15 second centrifuge. Each sample was then transferred to a Qiagen Mini-Column (Qiagen, Germany) and centrifuged at 6,000G for 1 minute. The Mini-Column was then placed into a Qiagen Collection tube (Qiagen, Germany) and 500µL of Buffer AW1 (Qiagen, Germany) was added. This was centrifuged at 6,000G for 1 minute. Next the Mini-Column was placed into a new Qiagen Collection tube and 500µL of Buffer AW2 (Qiagen, Germany) was added. The samples were centrifuged at 17,000G for 3 minutes before being placed into another new Qiagen Collection tube and being centrifuged at 17,000G for 1 minute.

Elution

For the elution of the sample DNA, each Mini-Column was placed into a 1.5mL Eppendorf tube and 200µL of molecular grade DNA free sterile water was added. This was left at room temperature for 5 minutes before being centrifuged at 6,000G for 1 minute. The resultant eluate was collected and stored as DNA CYST001.1.

The Mini-Column was then placed into another fresh 1.5mL Eppendorf tube and a further 100µL of molecular grade DNA free sterile water was added. Again this was left at room temperature for 5

minutes before being centrifuged as before. The resultant eluate from this second collection was labelled as DNA CYST001.2. Both eluates were stored at -20°C and their location recorded on LIMS.

As can be seen from the protocol above this resulted in two elutes for each sample. These eluates were then analysed using the Nanodrop Reader (Thermo Scientific, Boston, USA) and the resultant concentrations of DNA recorded as ng of DNA / µL of eluate.

The resultant concentrations for each elute of CYST001 – CYST 072 are recorded in Appendix A. The median concentration of extracted DNA was 43.25ng/µL (IQR: 19.8 – 77.3). There was no significant difference between the concentrations obtained from first or second elute (p=0.944).

For the remainder of the thesis it can be assumed that where DNA analyses took place on a sample, the eluate with the highest concentration of DNA was used.

4.3.2- Assessing Concentration

Throughout my lab work I have needed to assess the concentration of DNA within my samples – two methods were used; NanoDrop (ThermoScientific, Boston, USA) and Qubit (Life Technologies, New York, USA). Initially, until the purchase of the Qubit I used only the NanoDrop to assess the concentration of DNA extracted from the frozen samples.

NanoDrop uses UV absorbance measurements of samples, at 260nm to quantify DNA and the 260/280 ratio to estimate purity (quality) of nucleic acid – but is unable to differentiate between DNA and RNA.[244, 245] Furthermore at low concentrations the NanoDrop has been shown to be inaccurate by significantly overestimating the measurement; reliability can only be assured at sample concentrations of between 2 ng/µL to 15 µg/µL.[246, 247]

Conversely the Qubit uses Fluorometry based upon dyes which bind specifically with either DNA or RNA.[247] Most importantly to my work the Qubit has shown to be accurate down to concentrations of 10pg/µL.[247]

Qubit Flourometer

It is important that during the analyses of concentration only thin-walled, clear 0.5mL PCR tubes are used so as not to impede the flourometer. The reagents provided should be kept refrigerated at 4°C (with the exception of the dye) and then allowed to reach room temperature prior to use. The following protocol was followed:

1) The number of thin-walled clear PCR tubes needed was calculated as follows: n+2 (where n= the number of samples to be assayed).
2) The 'working solution' was made by diluting the Quant-iT (Life Technologies, Boston, USA) reagent 1:200 into the Quant-iT buffer. 200µL is required for each tube.
3) Into the first two tubes 190µL of working solution was placed, 195µL was placed into the remaining sample tubes.

4) In the first tube 10µL of Standard 1 (Life Technologies, Boston, USA) was added and this was labelled as ‘standard 1’.
5) In the second tube 10µL of Standard 2 (Life Technologies, Boston, USA) was added and this was labelled as ‘standard 2’.
6) Each of the remaining tubes had 5µL of individual sample added and were then labelled.
7) All tubes were then vortexed for 3 seconds.
8) The Qubit was calibrated by selecting ‘calibrate’ from the menu and inserting Standards 1 and 2 when prompted.
9) Each sample was then inserted in turn into the flourometer using the ‘calculate’ function to record the concentration of DNA in ng/µL.

4.3.3 - Limiting Dilution

Theory

Limiting Dilution describes the process of reducing the concentration of genomes in a sample to try to increase the concentration of mutant genomes within an aliquot to increase the sensitivity of detection at PCR.

Only approx. 1% of genomes from my samples may be mutant – PCR will introduce an error of ~1% so how do we distinguish the PCR error from genuine mutants?

Conventional PCR would not be sensitive enough to distinguish 'true' mutants from PCR inferred error. If we start with a definite number of genomes we can expect that 'true' mutants would be amplified at the same ratio as they appear in the sample.

If we accept a minimum of 1 in 10 (10%) and ensure each sample is 10 genomes we should see mutants of ≥10% of total reads where the mutant sequence is present and 0% otherwise.

If we take PCR error in the region of 1% any mutations <10% can be ignored as erroneous.

For illustration assume that of 100 genomes of p53, 1 is mutant. If I take 10 non-sequential dilutions of this down to 10 genomes per sample there will likely be at least 1 dilution which contains a mutant genome. This dilution then has 1 mutant out of 10 genomes (10%), compared to the original sample of 1 per 100.

PCR of each separate dilution will introduce a 1% error in calling 'false' mutations. It can be seen that if the original 'stock' solution was used the 1% error rate would mask the 1% 'true' mutation rate. Using the limiting dilution method 10 samples are created, one of which will probably have a mutant sequence and in this sample there will be a mutation ratio of 10% thus, whilst the remaining 9 samples may appear as wild type, there will be a 10% mutation read alongside the background 1% error rate.

It is analogous to try to blindly select a single black snooker ball from a bag which contains 99 red balls. If you are allowed to select a ball 10 times (replacing the ball each time) there is a probability of 0.01 that you will select the black ball (1:99). If the sample of 100 balls is randomly placed into 10 separate bags and you can select one ball from each bag the probability is now 0.1 (1:9) that you will select the black ball in one of the bags.

What is 10G?

A genome is defined as one complete copy of an organism's DNA. As humans have diploid cells, each cell will contain two copies of *TP53*. I aimed to ensure that only 10 copies of sample genome were present in each well of the PCR reaction.

Before I could begin the process of diluting my samples down to 10G I needed to try to ensure that they were all at the same starting concentration – real-time PCR was used to analyse my samples for their concentration against a purchased sample of standardised 10G human DNA (Sigma, 2ug). I was aware that whilst each sample likely contained millions of genomes I needed to ensure that I did not inadvertently dilute below 10G during this first step. The mass of 10G of human DNA was calculated using the following assumptions:

A. Number of base pairs in haploid human genome = 3^{10^9}
B. Average molecular mass of 1 base pair = 660 Daltons
C. 1Da = $1.67^{10^{-27}}$g

This means that a single genome of human diploid DNA weighs $6.6^{10^{-27}}$g or 6.6pg (((AxB)x2)xC). Thus 10G = 6.6pg x10 = 66.6pg. As long as the concentrations did not fall below 66.6pg/µL at least 10G would be likely.

Standardisation

Each sample of extracted DNA had been analysed using the NanoDrop (Thermo Scientific, Boston, USA) to ascertain the concentration of DNA in each eluate. As previously described, each sample

underwent two elutions (p.523) and the more concentrated of these elutes was taken for the following steps.

The PCR protocol (p.68) would require a maximum of 5µL of sample DNA and that there were 8 Exons, each with a forward and reverse reaction for the ion torrent. Furthermore, each reaction would need to be repeated 10 times, thus I needed a minimum of 800µL (8x2x10x5µL). A 1mL stock of each sample was prepared at a concentration of 0.25ng/µL. Where necessary serial dilution techniques were employed to ensure that 0.25ng of each sample was placed into a sterile Eppendorf and reconstituted with sufficient volume of molecular grade DNA free sterile water to result in 1mL. I randomly selected ten samples and confirmed the concentration was 0.25ng/µL using the Qubit (Life Technologies, New York, USA) analysis (p.55). The median concentration was 0.25ng/µL (error +/- 0.02ng/µL).

From now on all samples referred to as CYST0-- can be considered as being the 0.25ng/µL dilution of the original.

Dilution Factor

As 10G is a constant for each sample a constant factor can be calculated which could be applied to each sample to create a sample of an appropriate volume at a concentration of 66.6pg/µL.

I performed *KRAS* light cycler analysis on two randomly selected samples (CYST003 and CYST012) and compared them to a known sample at 10G using the following protocol:

1) 10µL of LC480 SYBR Green (Roche Diagnostics, Mannheim, Germany)
2) 0.5µL of Forward and Reverse Primers for *KRAS* (Appendix B)
3) 2µL of sample (either 10G standard, CYST003, CYST012, or Molecular Grade DNA free sterile water)
4) 7µL Molecular Grade DNA free sterile water

I performed the analysis twice.

The cycle threshold for the 10G standard was 46 compared to 41 for CYST003 and 40 for CYST012.

Therefore, as each cycle represented a doubling of the DNA present I diluted CYST003 to 1:25 (5^2) which represented 5 cycles (46 – 41) and CYST012 to 1:36 (6^2) which represented 6 cycles (46 – 40).

I then repeated the experiment using exactly the same protocol but with the new dilutions of CYST003 and CYST012 included as well as the original 0.25ng/µL samples. The results can be seen in Table 6.

Table 6: Comparison of two samples against known 10G standard.

Sample	Cycle Threshold at Original Concentration	Cycle Threshold of Diluted Sample
10G	44 cycles	-
CYST003	42 cycles	(1:25) 53 cycles
CYST012	39 cycles	(1:36) 48 cycles

This is an example of samples being over-diluted and so the process was repeated using 1:4 dilution of CYST003 and 1:25 of CYST012 (Table).

Table 7: Second comparison of two samples against known 10G standard.

Sample	Cycle Threshold at Original Concentration	Cycle Threshold of Diluted Sample
10G	44 cycles	-
CYST003	42 cycles	(1:4) 44 cycles
CYST012	39 cycles	(1:20) 44 cycles

The concentration of DNA using the Qubit (p.55) was ascertained for each of these diluted samples, I then divided this by the required concentration needed to take them from 0.25ng/µL to 10G (44 cycles) giving a constant 'dilution factor'. This can be expressed as:

$$\frac{[DNA]}{\text{Dilution}} = \text{Dilution Factor (DF)}$$

Thus, the exact volume required to obtain 10G was calculated for the remaining samples using [DNA] x DF.

The Qubit concentration of CYST003 was 0.267ng/µL and CYST012 was 0.663ng/µL.

0.267ng/µL ÷ 4 cycles = 0.06675

0.663ng/µL ÷ 20 cycles = 0.03313

The average of these values is 0.04994, rounded to give a Dilution Factor of 0.05.

Preparation of Samples

Each of the samples (at 0.25ng/µL) was diluted by 0.05 x their Qubit confirmed concentration, e.g. CYST073 had a concentration of 0.2610 – when multiplied by 0.05 this meant that 0.013µL contained 10G.

Once I had optimised the PCR reaction (p.68) I knew that I would be using 4µL of sample per reaction, so there needed to be a final concentration of 10G per 4µL. I opted to create 1mL of each sample to allow for spillage/error/repeats/etc.

Worked example using CYST073.

- Initial concentration 0.2610ng/µL x DF 0.05 = 0.013µL would contain 10G.
- To make 4µL at 10G would require 0.013µL of CYST073 + 3.987µL of molecular grade DNA free sterile water.
- To make 1mL would require 250 x 0.013 (1000µL ÷ 4µL) = 3.25µL.
- Thus 996.75µL of molecular grade DNA free sterile water (1000µL – 3.25µL) needs to be added.

The above dilutions were made to every sample and accordingly labelled as CYST001/10G; CYST002/10G and so on.

4.3.4 - PCR Optimisation

Dr Li Yan had already optimised a PCR programme for *TP53* using her own primers (to which I had added barcodes and ion torrent adaptors, p.50) so the initial protocol was adapted from her protocol for a 25µL reaction:

- 2.5µL of 10x Buffer (Envitrogen LifeScience Technologies, New York,USA)
- 1.5µL of MgCl (25nM) (Envitrogen LifeScience Technologies, New York,USA)
- 0.52µL of dNTP (KAPA Biosystems, KAPA dNTP Mix,Mixture of dATP, dCTP, dGTP and dTTP, 10 mM each)
- 0.129µL of Apmli-Taq Gold (Envitrogen LifeScience Technologies, New York,USA)
- 0.4µL of Forward and Reverse Primers (1 pMol)
- 17.49µL of molecular grade DNA free sterile water
- 2µL of DNA

The PCR was optimised using the DNA diluted to a concentration of 0.25ng/µL to avoid wastage. I started with one barcode primer from each exon. The PCR programme was automated using the following steps:

- Heated Lid
- Hot start
- 5:00 at 90°C
- Annealing - 70°C for 0:05
- Binding - 55°C for 0:05
- Melting - 95°C for 0:10
- 45 cycles
- 72°C Final 10:00

The resultant PCR product was then run on a 2% agarose gel (TAE Buffer + Agarose + Nucleic Acid Dye (Biotium, GelRedTM Nucleic Acid Gel Stain)) for 25 minutes at 130V / 400mA. Each well was loaded with 5µL product and 2µL of loading dye (Promega, Blue/Orange Loading Dye, 6X). For comparison 1.2µL of ladder (Roch, DNA Molecular Weight Marker XIII) was used.

There was a significant amount of primer-dimer, in fact it was impossible to identify the product which would have been expected at 180-205 bp.

The specificity of primers can be improved using the following methods:

1.) Decrease the concentration of pimers.

2.) Increase the annealing temperature.

3.) Decrease the concentration of MgCl.

Thus using the same DNA extraction and primers (5F1A2.5; 6FA3.5; 7F1P3.4; 8F2A2.4) I set four separate PCR reactions with water controls, altering only the following parameters and maintained everything else constant:

1.) a.) Primer dilution 1:10.

b.) Primer dilution 1:100.

2.) Annealing temperature increased to 60°C.

3.) MgCl volume decreased to 1.0µL (the difference made up with water).

The resulting 1.8% Agarose gel can be seen in Figure 4. Appropriate bands can be observed for Exons 7 and 8 at a dilution of 1:100 primers, annealing temperature of 55°C and MgCl (25nM) volume of 1.5µL. Unfortunately the control wells were also contaminated with DNA, but at least this proved that the primers were indeed amplifying product. There were no bands produced for Exons 5 or 6 using any of the variations above. When I repeated exactly the same experiment using more primers from Exons 7 and 8 I did not produce any PCR product – the only discernible difference in the protocol was

that I had created the 'master mix' of sample DNA free PCR ingredients in the designated 'DNA Free' room in The University Clinical Department.

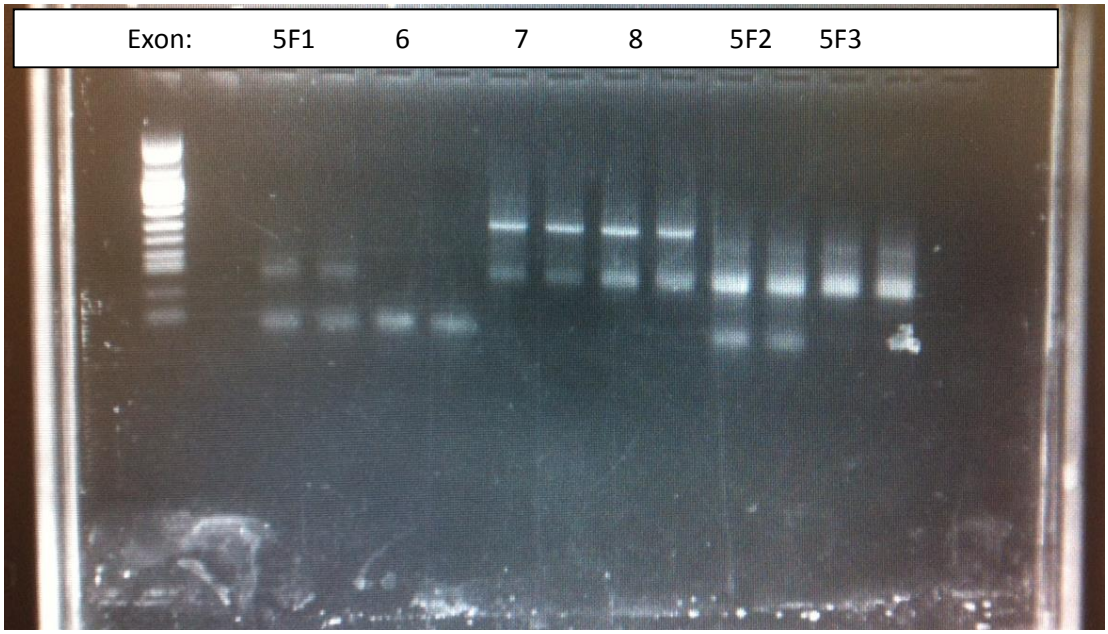


Figure 4: 1.8% Agarose Gel showing appropriate bands for Primers of Exon 7 (F1 and F2) and Exon 8 (F1 and 2) but not Exons 5 or 6.

Exons 5 and 6

I designed a PCR protocol for a touch-down PCR with an annealing temperature from 60°C-50°C in the hope that I may see bands of product suggesting the range in which I should search for the optimum annealing temperature for Exon 5 and/or 6. I also realised that the annealing temperature for exon 5 may be different from that required for exon 6, which would make future analysis all the more complicated in a 96-well plate format.

When I reviewed the gels from my first primer variable experiments, I noted that despite the lack of product there were strong bands representing primer-dimers in the wells of Exon 5 and 6 primers which were run with an annealing temperature of 60°C. PCR reactions were set up using an annealing temperature of 61°C, 63°C, 65°C. Primer-dimer was apparent at 61°C but there was no product at 63°C or 65°C. I ran a PCR protocol using primers from exon 5 and 6 at 62°C which revealed bands at the expected length for my product (Figure 5).

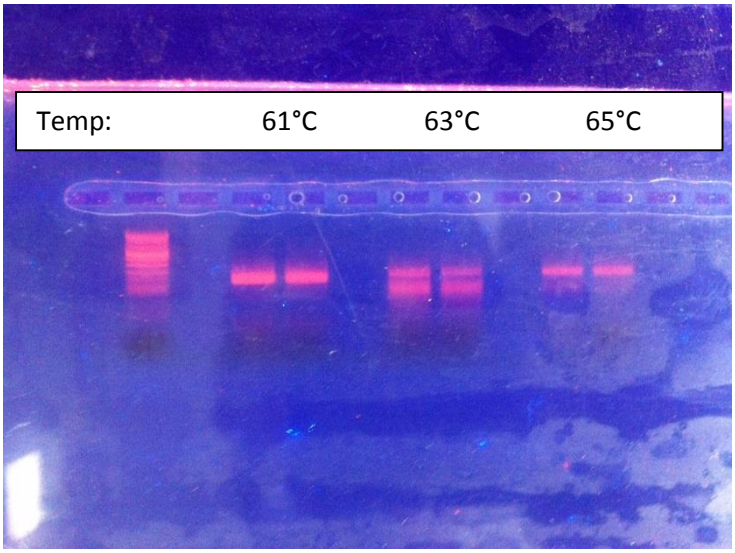


Figure 5: 1.8% Agarose Gel showing appropriate bands for primers of Exon 5 (F1, 2 &3)

I had now found the optimum annealing temperature for all 4 exons:

- Exons 5 and 6 = 62°C
- Exons 7 and 8 = 55°C

So two separate PCR runs for each sample would be needed.

Mass Production

I took one sterile 96-well plate (Star Labs, Manchester, UK) and in a DNA-free room I pipetted the volumes of each ingredient as listed on p.62 into the first 10 wells rows A-H. This plate became the PCR base for Exons 5 and 6 of sample CYST001. The process was repeated for a second 96-well plate which became the base for Exons 7 and 8. The plates were covered with adhesive aluminium foil and transferred to the main laboratory and 2µL of DNA from CYST001 (at 0.25ng/µL) was added to each well giving a total volume of 25µL. The plates were sealed and centrifuged at 6,000G for 15 seconds then placed into the thermocycler. The plate containing the primers for Exons 5 and 6 was set off using the protocol which included an annealing temperature of 62°C; the plate containing the primers for Exons 7 and 8 was set off using the protocol which included an annealing temperature of 55°C. The resulting agarose gels can be seen in Figure 6.

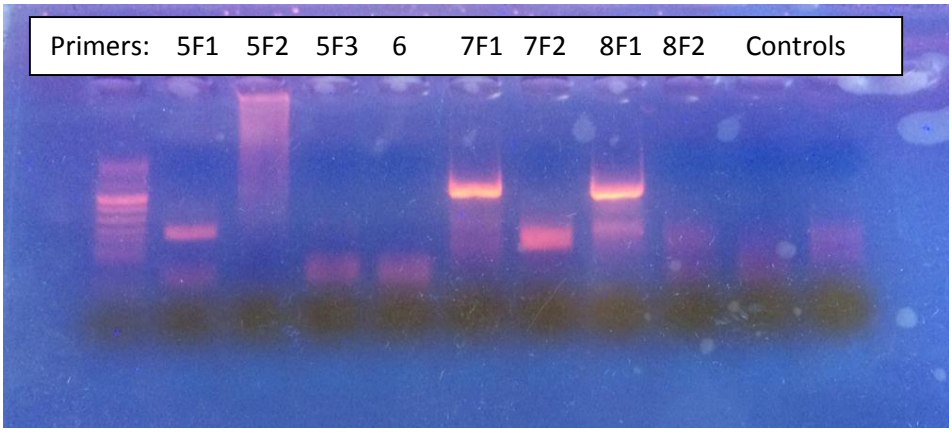


Figure 6: 1.8% Agarose gel showing scanty product for Exon 5 and 6 primers.

There was product in some wells but not in others which I found difficult to explain as the primers in adjacent wells were, by definition, only a single nucleotide different from those which had worked in my initial experiments.

There was room for pipetting error given the sheer quantity (160) wells which were being injected with PCR ingredients. Further none of the pipettes I had been using were 'negative-pressure' pipettes and so would not be accurate enough for ultra-low volumes such as those which I had been using for dNTP, primers and Taq.

I concluded that another approach to this problem would be to create a "mastermix" of all PCR ingredients (excluding sample DNA and primers) which could them be pipetted into each well with DNA added separately. This would also save me significant time. I calculated that I would need sufficient material for each barcode to be used 25 times (73 samples ÷ 3 barcode sequences). Each mix would therefore need the materials as detailed in Table 8.

This gave a total 'mastermix' volume of 556.756µL which could be pipetted into 25 wells and then have DNA added to it. I initially restricted this new mastermix method to Fragment 1 of Exon 5, Figure 7 shows that this method did successfully create product.

Table 8: Mastermix volumes.

Ingredient	Initial Volume	X 25
10x Buffer	2.5µL	62.5µL
MgCl (25nM)	1.5µL	37.5µL
dNTP (10nM)	0.52 µL	13µL
Apmli-Taq Gold	0.129µL	3.255µL
Forward and Reverse Primers (1pMol)	0.4µL	10µL
Molecular grade DNA free sterile water	17.49µL	437.25µL

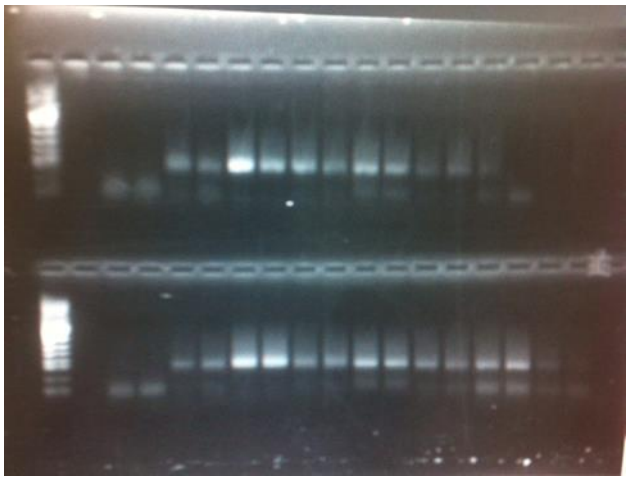


Figure 7: 1.8% Agarose Gel showing product for all primers. First two wells on each row are controls, the test wells are: 5F1forward; 5F1reverse; 5F2forward; etc…

In order for this method to work I would need to create 480 separate mastermixes – this posed issues in terms of the storage space within the DNA-free room and the concern that if I contaminated one of these mastermix vials then the whole volume (sufficient for 25 reactions) would have to be wasted.

This concern was compounded as dNTPs will degrade with multiple episodes of defrosting and re-freezing. Also I had presumed that the AmpliTaq gold would remain stable and inactivated until it was heated to 96°C, however, as the enzyme is stabilised in glycerol dilution with the other ingredients (especially the molecular grade water) could alter the properties and affect the quality of the Taq. On the basis of these factors I decided not to create these large volume mastermixes.

The compromise I attained was to dilute the primers with molecular grade DNA free sterile water so that I could pipette a larger volume into each reaction – this would be far easier than trying to

accurately pipette 0.4µL into each well. The forward primers only were diluted such that a volume of 1µL could be added to each well.

The Final Protocol

Within the DNA free room three 96-well plates were prepared by being placed under a UV light for 30 mins. Each of these plates was labelled with indelible ink – e.g. CYST001 Exons 5 & 6; CYST002 Exons 5 & 6; CYST003 Exons 5 & 6.

8 Sterile DNA free Eppendorf tubes were placed into a rack and labelled 1 – 8.

The following ingredients should be added to each of the eight tubes in turn:

1.) 80µL of 10x Buffer.

2.) 48µL of MgCl (25nM).

3.) 16.64 µL of dNTP (10nM).

4.) 540.48µL of molecular grade DNA free sterile water.

The tubes had 12.8µL of reverse primers added in the following order:

Tube 1 = Exon 5 F1 P – Reverse

Tube 2 = Exon 5 F1 A – Reverse

Tube 3 = Exon 5 F2 P – Reverse

Tube 4 = Exon 5 F2 A – Reverse

Tube 5 = Exon 5 F3 P – Reverse

Tube 6 = Exon 5 F3 A – Reverse

Tube 7 = Exon 6 P – Reverse

Tube 8 = Exon 6 A – Reverse

Finally to each of these tubes 4.128µL of AmpliTaq Gold was added – it is important to add this last to try and reduce the incidence of primer-dimer. The resulting tubes will have sufficient volume for 32 reactions; only 31 reactions are required so there should be a sufficient margin for error.

Using Tube 1, 22µL of the mixture was pipetted into the first 10 wells of row A in each of the three plates. Discard the tube. On the first plate column 11 was used as well, this was to be used as a **control** for the mastermix. I repeated this process for the remaining seven tubes in rows B – H respectively.

Using the pre-diluted forward primers 1µL of Exon 5 F1 A 1.1 was added to the plate labelled CYST001 in well A/1.

1µL of Exon 5 F1 A 1.2 was added to well A/2. This was continued along the row to Exon 5 F1 A 1.10, then continued along the next row with Exon 5 F1 P 1.1 in B/1. The process was further completed until I reached Exon 6 P 1.10 in H/10.

The process was repeated for the plate labelled CYST002 this time using the forward primers prefixed with 2, e.g Exon 5 F1 A 2.1 – 2.10, and for the plate labelled CYST003 this time using the forward primers prefixed with 3, e.g. Exon 5 F1 A 3.1 – 3.10.

Into column 11 of the first plate I added 2µL of molecular grade DNA free sterile water to act as a **control**. The eight wells in column 11 had corresponding forward primers added – these were randomly chosen from any of the barcoded primers which corresponded.

Each of the plates were covered with a sterile aluminium (heat-proof) cover and transfered to the DNA lab.

2µL of DNA at 10G was added to each of the wells in columns 1-10. The DNA corresponded to the label on the plate, CYST001, CYST002, etc. Once the DNA was added the plates were re-covered and placed into the thermo-cycler and set off on the pre-loaded programme called JIMBOB EXONS 5 & 6 (i.e. with an annealing temperature of 62°C).

This will provide PCR product and control reactions for Exons 5 & 6 for three samples.

Once the PCR reaction had completed the plates were stored at 4°C.

The entire process was repeated again this time for Exons 7 & 8. This time the reverse primers were applied as below:

Tube 1 = Exon 7 F1 P – Reverse

Tube 2 = Exon 7 F1 A – Reverse

Tube 3 = Exon 7 F2 P – Reverse

Tube 4 = Exon 7 F2 A – Reverse

Tube 5 = Exon 8 F1 P – Reverse

Tube 6 = Exon 8 F1 A – Reverse

Tube 7 = Exon 8 F2 P – Reverse

Tube 8 = Exon 8 F2 A – Reverse

The forward primers were applied as Exon 7 F1 A 1.1 – 1.10 and so on until Exon 8 F2 P 3.10.

A complete list of barcoded forward primers and their corresponding reverse primers is given in Appendix C.

4.3.5 - Extraction and Purification

Over the previous pages I have described the protocol for the preparation of the PCR mixture. As a result of the PCR there were six 96-well plates labelled as follows:

CYST001 Exons 5 & 6, CYST001 Exons 7 & 8 – Barcoded primers prefixed with '1'.

CYST002 Exons 5 & 6, CYST002 Exons 7 & 8 – Barcoded primers prefixed with '2'.

CYST003 Exons 5 & 6, CYST003 Exons 7 & 8 – Barcoded primers prefixed with '3'.

Each sample therefore had 160 wells of product, each containing 25µL. The first step was to confirm that the PCR has worked by running an agarose gel to confirm product was present. Thereafter this product needed to be extracted from the mixture and prepared for IonTorrent analysis.

Agarose Gel

Using a gel made of 1.8% agarose (Star Labs, Manchester, UK) and TAE buffer, I ran multiple samples in one electrophoresis reaction. Using the largest tank available, and the narrowest comb, I created a gel that was capable of holding 80 samples with marking ladders.

The settings for the gel electrophoresis were:

400mA and 130V for 25 minutes.

For each of the gels I used 5µL of PCR product to confirm that the PCR reaction had been successful; this left me with 20µL for further purification. In order to optimise my samples for the ion torrent I followed the published protocol provided with the torrent device – a technique known as AMPure (Life Technologies, New York, USA). Once I had confirmed the presence of appropriate bands on the 'check' gel I used the AMPure kit to prepare the DNA libraries ready for the ion torrent.

AMPure Clean-up

10µL of DNA product from each well was placed into a sterile 0.5mL Eppendorf.

I submitted the bottle of AMPure XP magnetic particle solution to a vortex to ensure the magnetic particles were fully suspended in the solution. (Each bead is made of polystyrene surrounded by a layer of magnetite, which is coated with carboxyl molecules. It is these that reversibly bind DNA in the presence of polyethylene glycol and salt. PEG causes the negatively-charged DNA to bind with the carboxyl groups on the bead surface).[248]

I then added 18µL (i.e. 1.8x volume) of AMPure XP magnetic particle solution to each Eppendorf and mixed well by re-pipetting 10 times. This was then incubated at room temperature for 10 minutes before being placed into the AMPure magnet (Figure 8) holder for 5 minutes (maximum of 16).

Fragments of DNA with a particle size of >100bp were attached to the beads so the supernatant was aspirated and discarded and each cluster of adherent magnetic beads was washed with 60µL of 70% ethanol which was left in place for 30 seconds before being aspirated and discarded. This was repeated once more to give a total of 2 washes. I then pipetted 15µL of 'Elution Buffer' (Beckman-Coulter) into each Eppendorf and mixed by pipetteing 10 times. The Eppendorfs were removed from the magnet and left to incubate at room temperature for 2 minutes.

The DNA fragments were now eluted from the beads and into the solution. The final step involved placing the Eppendorfs back onto the magnet for 1 minute and the supernatant (10µL to ensure no inadvertent aspiration of beads) was aspirated and transferred to a separate Eppendorf. This was then stored at 4°C in preparation for processing.

As can be seen this is a laborious procedure which requires a lot of consumables (Eppendorfs and sterile pipette tips) but also carries a significant risk of accidental cross-contamination. As only 16 Eppendorfs could be processed at a time it became apparent that this step may well become a rate limiting stage in the completion of my lab work.

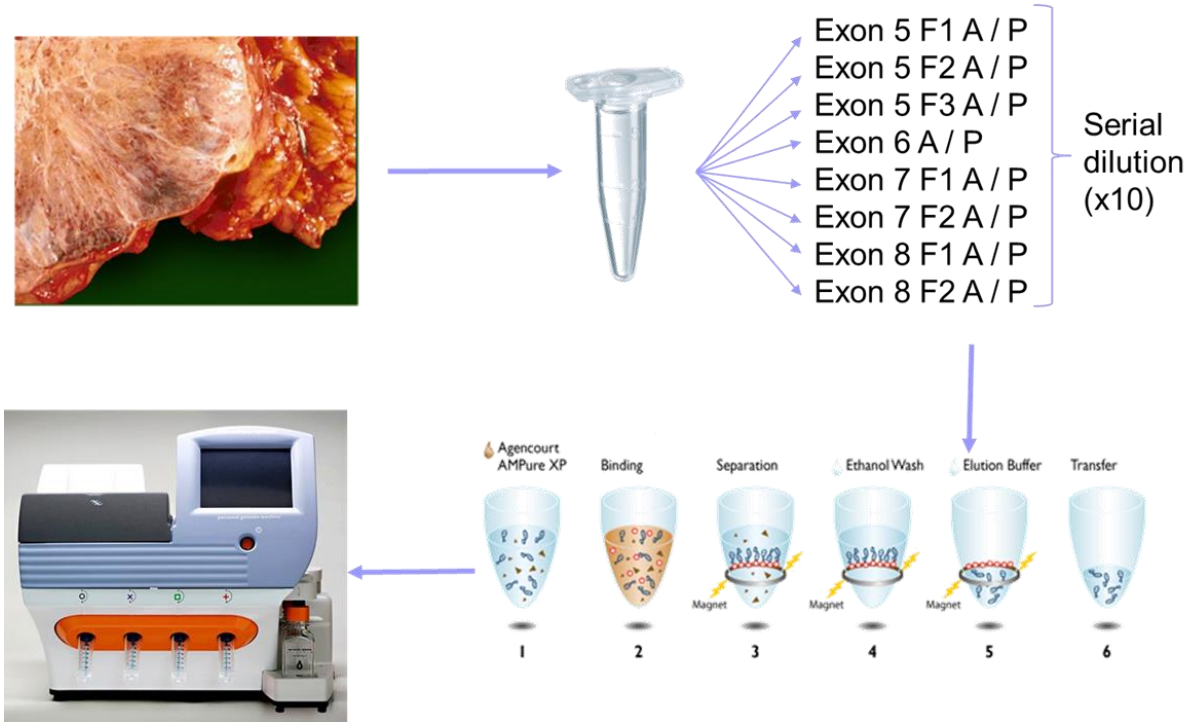


Figure 8: A pictorial schematic of Ion Torrent Methods.

Gel Purification

As I progressed through the purification of each of the PCR products from the first six 96-well plates I realised that I needed to find a way to speed up the process. When the first round of ion torrent data became available from those three samples it was apparent that, despite my efforts at AMPure purification, there was still significant contamination with primer-dimer – the first run of the ion torrent revealed that the chip had preferentially sequenced runs of primer-dimer rather than the product. I realised that this was most likely due to the fact that because of the addition of ion torrent adapters and barcodes the size of my primer-dimers would be in the region of 100bp, and thus they could not be adequately separated from the product during the magnetic bead purification.

There were two options:

1.) Decrease the ratio of AMPure beads to DNA from 1.8 to 0.8 which should increase the specificity of the procedure such that only fragments >200bp would be isolated, or:

2.) Find an alternative method, such as gel purification.

The mechanism is simple – the product for the PCR reaction is run on an agarose gel as long as is necessary for the product and the primer-dimers to separate. Then the agarose gel containing the DNA fragments of interest (the product) was cut out with a scalpel and the DNA was extracted using the following method:

Each cut band of agarose gel was weighed in a sterile Eppendorf and then 300µL of Agarose Solubilisation Buffer (Roche, Hamburg, Germany) was added for every 100mg of agarose. I then added 10µL of Silica Suspension (Roche) and then each Eppendorf was mixed using a vortex until a homogenous suspension had been created. The Eppendorfs were then incubated at 60°C for 10 minutes with each sample being vortexed every 3 minutes. Each Eppendorf was then centrifuged at maximum speed for 30 seconds – the supernatant was discarded. I added 500µL of Nucelic Acid Binding Buffer (Roche) to each Eppendorf and mixed on a vortex for 10 seconds before centrifuging at maximal speed for 30 seconds and discarding the supernatant.

Each pellet was re-suspended with 500µL of Washing Buffer (Roche) and mixed on a vortex for 10 seconds before another round of centrifugation at maximal speed for 30 seconds again discarding the supernatant. The washing step was repeated with 500µL of Washing Buffer.

The samples underwent centrifugation again at maximal speed for 30 seconds and the supernatant was dicarded. A final centrifugation was performed at maximal speed for 1 minute and any liquid carefully disposed of.

Each Eppendorf was carefully inverted onto an absorbent piece of tissue and left to dry at room temperature for 20 minutes. 30µL of AMPure Elution Buffer (Life Technologies) was used to suspend each pellet using the pipette to mix. The samples were then incubated at 60°C for 10 minutes, with a vortex being performed on each sample every 3 minutes.

Finally the samples were each centrifuged at maximal speed for 30 seconds and 25µL of supernatant (containing the extracted DNA) was transferred to a sterile Nunc tube and labelled accordingly. The samples were then stored at 4°C.

Refinements to Gel Extraction

Initially the protocol from Roche had called for distilled water to be used as the extraction solvent, however, I was aware that the Elution Buffer supplied by Life Technologies was specifically adapted for the ion torrent and so I sought to use it where possible. To ensure that the reagent did not interact negatively with any of the chemicals used in the process I performed a side by side comparison of the Elution Buffer versus distilled water over 4 random samples (Table 9).

Table 9: Examples of DNA concentration when elution is performed with water vs. elution buffer.

Sample	Elution with 30µL Buffer (ng/µL)	Elution with 30 µL Water (ng/µL)
5 F2 A 1.1 (CYST001)	0.240	0.0972
5 F2 P 2.6 (CYST002)	0.222	0.744
5 F3 A 3.4 (CYST003)	0.370	-
6 A 2.5 (CYST002)	0.240	-

On this basis I elected to use the ion torrent Elution Buffer as it gave more consistent results than distilled water.

Whilst this method was certainly quicker than the AMPure technique there was still the significantly labour intensive step of creating the large agarose gel on which I had to run each PCR well separately and then cut each band by eye.

It occurred to me that as long as I ensured that barcoded primers from different samples didn't mix I would be able to run a smaller number of wells by combining the PCR product for each barcode 1-10 of each exon.

Therefore once I retrieved the 96 well plate from the thermocycler at the end of the PCR run I could combine 10µL from each of the 10 barcodes for Exon 5 F1 A to create a single 100µL solution which

represented Exon 5 F1 A for that particular sample. If I did the same for each of the other rows on the plate I would end up with sixteen 100µL combinations for each sample.

These were then added to loading dye (5µL) and pipetted into two adjacent troughs on the agar gel. From now on I could run three entire samples on one single gel using this 'combination' method with far fewer extractions needed to prepare the libraries.

Even with these modifications it became apparent that I would need a significant volume of Gel Solubilisation Buffer, Nucleic Acid Binding Buffer and Washing Buffer from Roche. Purely in an effort to reduce costs I researched the contents of the kits using the manufacturers data sheets and used the following formulae to create two of the reagents from stock chemicals. The silica matrix still needed to be purchased however.

Gel Solubilisation Buffer = 6M NaCl + 0.05M Tris-Cl + 10mM EDTA.

Washing Buffer = 20mM Tris-Cl + 2mM EDTA + 0.4M NaCl + 50% v/v 99% ethanol.

4.3.6 - Preparing the Libraries

Once the DNA intended for sequence had been extracted from the agarose gel it needed to be prepared for use in the Ion Torrent Sequencer. This was achieved in three stages which can be summarised as: Concentration; Combination; and Calculation.

The overriding principle is to ensure that the distribution of each fragment of replicated DNA is equimolar across the chip – thereby ensuring that no single fragment dominates and will be preferentially sequenced at the expense of others.

Each fragment is thus presented to the Ion torrent as if were an individual patient – thus I am provided with 10 independent sequences (barcodes) of each fragment for each of the three patients on the chip.

Concentration

Each sample which had been extracted from the gel, a volume of 25µL, was inserted into the Qubit to ascertain the concentration of DNA for each of the fragments 1-8, this was then noted in a table (e.g Table).

Table 10: Example of DNA concentrations after gel extraction.

Patient e.g. CYST001	Concentration (ng/µL)
Exon 5 Fragment 1	1.22
Exon 5 Fragment 2	0.97
Exon 5 Fragment 3	1.78
Exon 6	1.37
Exon 7 Fragment 1	1.04
Exon 7 Fragment 2	0.76
Exon 8 Fragment 1	1.67
Exon 8 Fragment 2	1.08

Three patient samples (CYST001 – CYST003) were analysed on each occasion, providing a total of 24 concentrations. I then combined the samples to result in one single sample for the Ion Torrent to sequence.

Combination

In order to combine the samples in an equimolar distribution I first needed to calculate the concentration of each sample in pM using the following formula:

$$\text{pM} = \left(\frac{\text{Sample Concentration (ng/µL)} \times 10^9(\text{µL/L})}{660\text{g x Size of Fragment (bp)}}\right)$$

Then, using the value of the lowest concentration in each sample, the following calculation is performed:

Lowest concentration of Patient (ng/µL) ÷ Concentration of each sample (nM)

The resulting value is the volume in microlitres of each sample required to result in the same concentration in nM as is present in the most dilute. A worked example is shown in Table 11 .

Table 11: An example of calculations required to establish volume of each sample needed based on concentration.

Exon	bp	PCR Conc (ng/µL)	pM	nM	Volume (µL)
E5.1	215	1.22	8597.60	8.60	0.09
E5.2	223	0.97	6590.57	6.59	0.12
E5.3	188	1.78	12094.03	12.09	0.06
E6	215	1.37	9308.33	9.31	0.08
E7.1	193	1.04	7066.18	7.07	0.11
E7.2	211	**0.76 (lowest)**	5163.75	5.16	0.15
E8.1	197	1.67	11346.65	11.35	0.07
E8.2	219	1.08	7337.95	7.34	0.10

As can be seen from the example above, I would be required to pipette volumes as low as 0.07µL which is not possible to do with accuracy, as a result I applied the same multiplication factor to each volume until all of the volumes were greater than 1µL, see Table 12.

Table 12: Serial volume increases required to establish adequate volume of final sample.

Exon	Volume (µL)	x2	x4	x6	x10	x20
E5.1	0.09	0.18	0.35	0.53	0.88	1.77
E5.2	0.12	0.23	0.46	0.69	1.15	2.31
E5.3	0.06	0.13	0.25	0.38	0.63	1.26
E6	0.08	0.16	0.33	0.49	0.82	1.63
E7.1	0.11	0.22	0.43	0.65	1.08	2.15
E7.2	0.15	0.29	0.59	0.88	1.47	2.94
E8.1	0.07	0.13	0.27	0.40	0.67	1.34
E8.2	0.10	0.21	0.41	0.62	1.04	2.07

This would therefore result in a 'combination sample' for this patient with a volume of 15.47µL. This combination sample would contain 10 barcoded PCR amplifications of each fragment of *TP53* in an equal distribution.

Once this was performed for all three patients I am left with 3 combination samples of varying volumes and concentrations depending, of course, on the lowest concentration within each one.

The process of combination was then repeated again after first ascertaining the concentration of each combination sample using the Qubit. The same formula is used, however as each combination sample contains 8 different fragments the value for fragment size within the calculation is changed to the average of all 8 i.e. 207.25bp.

Using this calculation I obtained the volume required from each of the combination samples to create an equimolar final library to place onto the chip. This final library contains the PCR amplified DNA from three different patients at a constant concentration.

Calculation

This 'final sample' was labelled as J1 which correlated with the chip it was to be run on. Thus J1 contained the first three patients: CYST001 -003; and J2 held CYST004 -006; etc…

In order to begin the Ion Torrent process the concentration of J1 was defined using the Qubit and this was used to determine the final dilution which would be used based upon the premise that the ion torrent chip will hold a maximum volume of 5µL which, in turn, should contain 40 million molecules for optimal sequencing.[a]

Clearly then, whatever the resultant concentration of J1 significant dilution was required – the factor of dilution was obtained using:

$$\text{Dilution Factor} = \text{Library Concentration (nM)} \times \left(\frac{5\text{x}10^{9}\ \text{molecules}/\mu\text{L}}{8.3\ \text{nM}}\right) \times \left(\frac{5\ \text{uL}}{40\text{x}10^{6}\ \text{molecules}}\right)$$

[a] *This is taken from the Instructions for Use of the Ion Torrent, provided with the software*.

Thus to calculate what starting concentration would **not** require a dilution:

$$\text{Library Concentration (nM)} = \left(\frac{5\text{x}10^{9}\ \text{molecules}/\mu\text{L}}{8.3\ \text{nM}}\right) \times \left(\frac{5\ \text{uL}}{40\text{x}10^{6}\ \text{molecules}}\right) \times 1\ (\text{i.e. no dilution})$$

$$\text{Library Concentration (nM)} = \frac{1}{\left(\frac{5\text{x}10^{9}\ \text{molecules}/\mu\text{L}}{8.3\ \text{nM}}\right) \times \left(\frac{5\ \text{uL}}{40\text{x}10^{6}\ \text{molecules}}\right)}$$

$$\text{Library Concentration (nM)} = \frac{1}{(6.02 \times 10^{8}) \times (12.5 \times 10^{-8})}$$

$$\text{Library Concentration (nM)} = \frac{1}{75.25}$$

$$\text{Library Concentration (nM)} = 0.01328$$

Using serial dilution of 5µL of J1 to 10µL of molecular grade DNA free sterile water the library can be sufficiently diluted to a concentration of 0.01325nM (i.e. 40 million molecules).

For Example:

If the concentration of J1 was 0.292ng/µL then 5µL combined with 10µL of water would result in a concentration of 0.973nM:

$$\text{nM} = \frac{(5\mu\text{L} \times 0.292\text{ng}/\mu\text{L})}{(5\mu\text{L} + 10\mu\text{L})}$$

If 5µL of this resultant mixture was again added to 10µL of water the resultant concentration would be 0.032ng/µL. Finally, by re-arranging the formula above, the volume of water required to reach a concentration of 0.01325nM can be calculated:

$$\mu\text{L} = \left(\frac{(5\mu\text{L} \times 0.032\text{ng}/\mu\text{L})}{0.01325\text{nM}}\right) - 5\mu\text{L}$$

$$= 7.216\mu\text{L}$$

5µL of this resultant dilution can therefore be used in the Ion Torrent reaction.

Chapter 5

RESULTS

5.1 – Results from the EUROPAC Registry

During my tenure as the EUROPAC fellow at Liverpool I was responsible, with Miss Sara Harrison, for maintaining the EUROPAC database in compliance with GCP guidelines. In this chapter I will relay the contents of the database for both FPC and HP, the definitions and descriptions of which can be found in my introduction.

Hereditary Pancreatitis

As discussed in the introduction, HP kindreds are recorded on the EUROPAC database under the mutation which they are proven to carry – or as Neg All, sporadic, CNV or familial HP if no PRSS1/CFTR mutation can be demonstrated. Table 6 displays the relative frequencies of these kindreds as well as the number of cases of confirmed PDAC which have been recorded.

Table 13: Relative frequencies of HP kindreds by type in the EUROPAC database.

Mutation	Families	Affected Individuals	PDAC cases
p.R122H	101	477	25
p.N29I/T	46	195	11
p.A16V	11	26	3
Other *PRSS1*	15	27	1
Neg All HP/CNV	49	181	18
Possible HP	62	106	3
Familial IP	67	126	27
CFTR	21	30	1
Sporadic	187	192	3
TOTAL	**559**	**1,360**	**92**

Familial Pancreatic Cancer

In the EUROPAC registry an FPC kindred is sub-defined as:

"True" FPC: Pancreatic cancer which has occurred within 2 or more first degree individuals across 2 or more generations (e.g. father and son). Within the EUROPAC cohort there are also kindreds in which there have been more than 2 cases of cancer. **267 kindreds with 662 cases of PDAC**.

"?" FPC: Kindreds in which there have been 2 or more cases of pancreatic cancer amongst first degree relatives within one generation (e.g. brother and sister). **100 kindreds with 225 cases of PDAC**.

"Other" FPC: This includes kindreds in which there have been at least one case of pancreatic cancer in conjunction with either; an associated mutation known to predispose to pancreatic cancer, or more than two cases of another cancer known to be associated with a predisposition to pancreatic cancer in the absence of any proven mutation (Table 14).

Table 14: Relative frequencies of FPC sub-type (with mutation or syndrome) where known, among EUROPAC kindreds.

Family Diagnosis	Number of Families
Breast-Ovarian	25
BRCA2	14
FAMMM	4
HNPCC	8
Possible HNPCC	6
Neurofibromatosis	2
Peutz-Jehgers	4
With gastric	17
FAP	1
Other	51

The geographical distribution of FPC and HP patients can be seen in Figures 9 and 10.

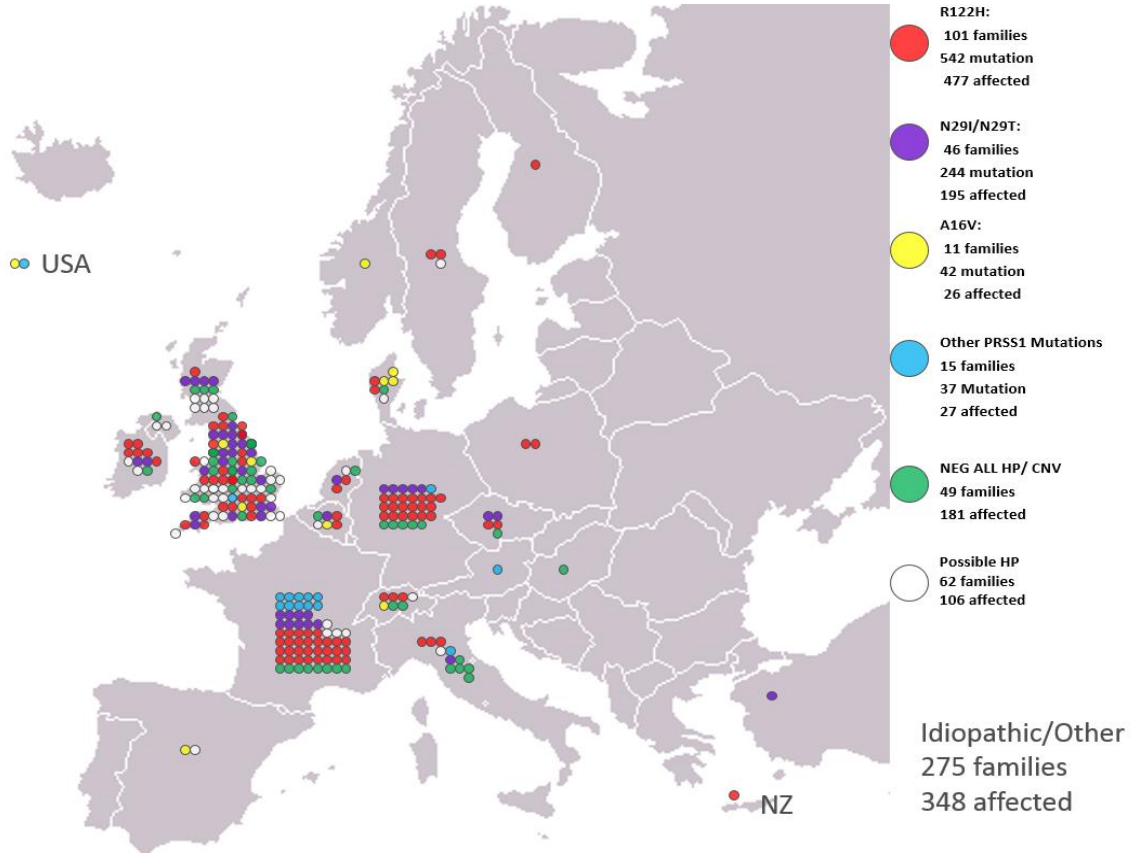


Figure 9: Geographical (Europe) distribution of HP families according to EUROPAC database.

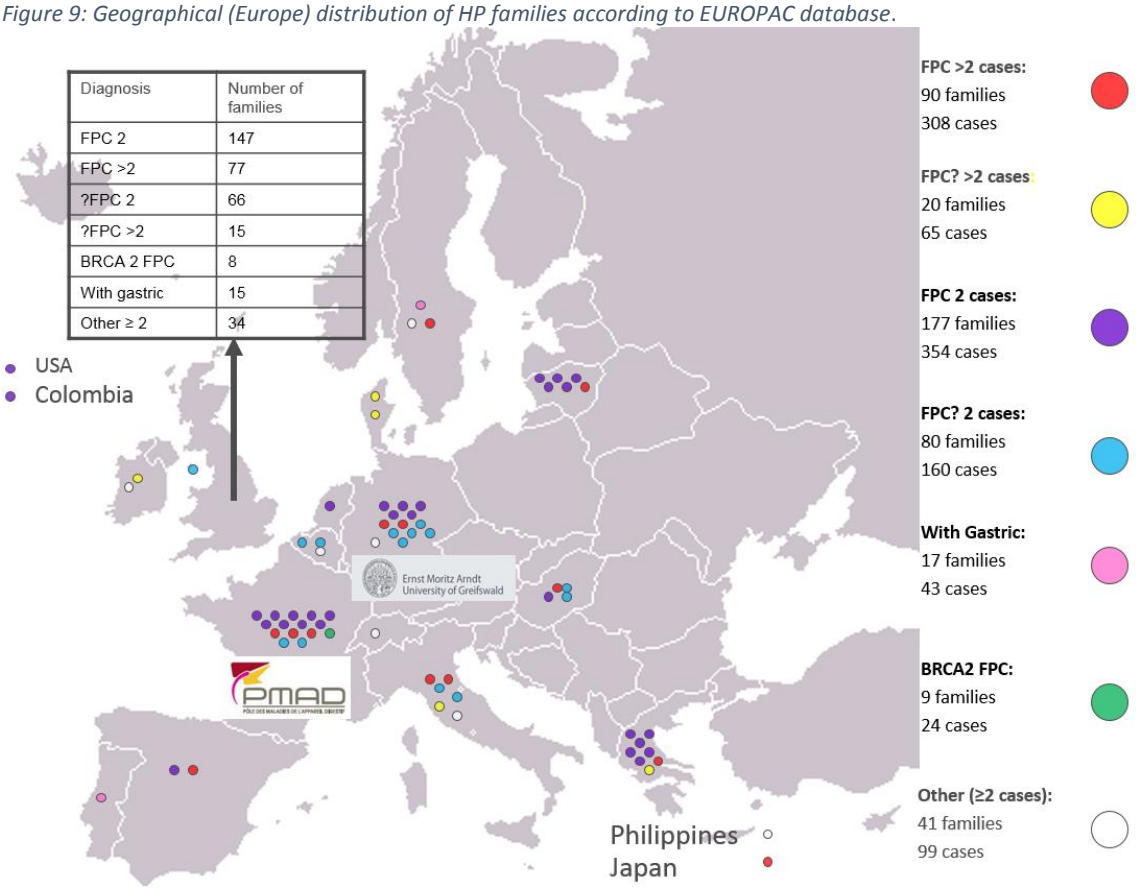


Figure 10: Geographical (Europe) distribution of FPC families according to EUROPAC database.

5.2 – Pilot Study Undertaken by EUROPAC

Between 1996 and 2005 the EUROPAC study was collecting data and tissue samples from individuals who underwent pre-operative ERCP prior to resection of pancreatic cysts which were felt on clinical and radiological grounds, to harbour malignant potential. The collection of this data began with Mr Nathan Howes and continued with each subsequent EUROPAC Fellow that followed him.

These data and specimens remained in storage at the Pancreas Biomedical Research Unit until 5 years had passed from the date of the last recruit. In 2010 work began to analyse the juice collected at ERCP and the tissue collected at the time of operation. I suggested we should start our search for a novel genetic predictor of survival with p53 and so Dr Li Yan analysed the samples using the previously described modified functional yeast assay.[139] I am presenting this as part of my thesis because I performed the analyses of the results and this serves to explain why my doctoral research took the direction that it did.

The following data was presented by me at the American Hepato-Pancreato-Biliary Association (AHPBA) in Miami, USA on 25/02/2012.

29 patients underwent partial resection of their pancreas for cystic neoplasm(s) in 9 years at a single institution (The Royal Liverpool University Hospital). 12 were male and 17 female. The median age at the time of resection was 66yrs (IQR: 23-79). All individuals underwent an ERCP pre-operatively with collection of pancreatic juice from the pancreatic duct stimulated by intravenous secretin administration at 1mg/kg.

The pancreatic juice obtained by direct aspiration was centrifuged and both pellet and supernatant were stored separately at -80°C.

The patients then went on to have their operative resection and samples of tumour were obtained from their resected pancreata by a consultant pathologist immediately prior to routine histological processing. These representative tissue samples were snap-frozen in isopentane and stored at -150°C.

After 5 years these tissue samples were defrosted and analysed for the presence or absence of a p53 mutation using the modified yeast assay in a blinded fashion.[139] The matched samples of supernatant and pancreatic juice were similarly processed.

Independent histological analysis was performed by a single consultant histopathologist without reference to the original histology reports provided at the time of resection. This was performed using original H&E stains from resected pancreata.

After this analysis 11 patients were diagnosed as having IPMN; 7 were IMPN with carcinoma in-situ (IPMC); 3 were PDAC; 6 were cystadeoma; and 2 were pseudocysts. For the purposes of future assessment these were grouped into 'Malignant' (IPMC and PDAC), n=10; and 'Benign', n=19.

p53 mutations were found in 9 tissue samples: 2 IPMN; 4 IPMC; 2 PDAC; and 1 cystadenoma. The survival data at 5 years is demonstrated in Table 15.

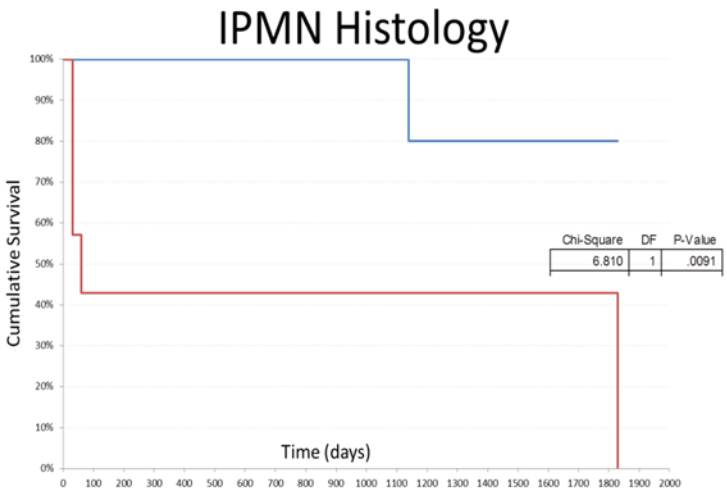
Table 15: Survival and mutational data from 29 patients who underwent operative resection 1996 – 2005.

Diagnosis (n)	Death from PDAC at 5 yrs (n)	P53 mutation in deceased / p53 mutation total
IPMN (11) - Benign	2	2/2
IPMC (7) - **Malignant**	5	4/4
PDAC (3) - **Malignant**	3	2/2
Cystadenoma (6) - Benign	0	0/1
Pseudocyst (2) - Benign	0	0/0

When grouped into 'malignant' and 'benign' it can be seen that 6 patients within the malignant cohort had a p53 mutation (60%) compared to 3 patients within the 'Benign' cohort (16%). Most striking of all the 2 patients with benign IPMN who had a p53 mutation went on to develop PDAC within 5 years; the remaining 9 patients who had benign IPMN with wild type p53 survived. Similarly, 5 of the 7 patients with IPMC died four of which had a p53 mutation.

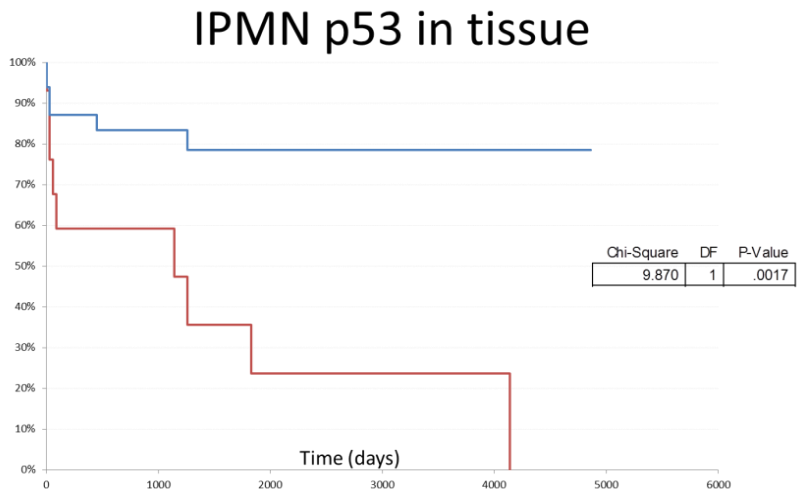
Overall survival was 780 days (IQR: 15-340) and there was no significant difference between median survival for the 'malignant' cohort (420 days; IQR: 15-1,800) and those patients with a p53 mutation (450 days; IQR: 15-1,800). However, when analysed using the Kaplan-Meier method (with the Mantel-

Cox log-rank test) it can be seen that mutant p53 was a more significant predictor of poor survival than histology alone (p=0.0017 vs. p=0.0091)(Figures 11 and 12).



Benign	11	7	7	6	4	1
Malignant	7	3	3	1	1	0

Figure 11: Kaplan-Meier analysis of survival for malignant versus benign cohorts



Wild Type	13	9	5	4	2	0
Mutant	5	2	1	1	1	0

Figure 12: Kaplan-Meier analysis of survival for p53 mutant versus wild type cohorts.

A Cox-regression analysis was performed including age, diabetes and gender as co-variants (smoking status was not available) which revealed that histology vs. survival had a coefficient of 0.221 (p=0.0819) compared with p53 status vs. survival; 15.073 (p=0.0152). Thus a mutation of p53

correlated with survival independently of and more significantly than the correlation between histology and survival.

The sensitivity and specificity of p53 status as a predictor of survival at 5 years was therefore calculated as 0.89 and 0.95 respectively.

It was also noted that in all cases where a p53 mutation was detected in the tissue a mutation was also detected in the juice sample analysed. In two cases, however, the mutation detected within the juice differed in genomic location from that which was detected in the corresponding tissue sample.

The conclusions of this (albeit small) study were threefold:

1.) p53 mutational analysis is not a surrogate marker for histology.
2.) p53 mutation is at least as good at predicting survival as eventual histological diagnosis in IPMN.
3.) p53 can be accurately detected in pancreatic juice.

The remainder of this thesis sets out to examine wether there is a single nucleotide polymorphism which can predict which IPMN harbour malignant potential, and possibly present an opportunity for gene therapy in the future.

5.3 - EUROPAC Screening Outcomes

When offering screening to individuals registered with the EUROPAC Study various options are available, the exact type of screening offered to an individual depends upon their physical status and risk as determined from their family history. Initially screening takes the form of a fasting blood sample to look for glucose impairment, followed by the offer of CA19-9 and a CT scan of the pancreas. In certain high risk individuals an ERCP is offered in order to obtain (via direct cannulation of the pancreatic duct) some pancreatic juice which is analysed for the presence or absence of *TP53* and *KRAS* mutations as well as the value of CDK2Na promoter methylation.

Pancreatic juice collection at ERCP carries a risk of post-ERCP pancreatitis (PEP) which can be potentially fatal. As those individuals undergoing screening are a healthy cohort with no clinical indication for ERCP it is imperative that all possible steps are taken to mitigate this risk. Studies have shown that the use of rectal diclofenac (a non-steroidal anti-inflammatory) reduces the incidence of PEP in individuals undergoing ERCP for clinical indications (gallstones, malignant biliary obstruction, etc…). Similarly the routine use of a short plastic stent inserted into the pancreatic duct after the procedure has been shown to improve outcomes.

This chapter of the thesis contains a paper in which we demonstrated that in our heathy cohort of screened individuals we can significantly reduce the incidence of PEP using a combination of these methods. The individuals referred to in this screening paper are EUROPAC registrants and not individuals from whom samples have been analysed as described in the methods section. I hope the inclusion of these data will demonstrate that screening using pancreatic juice is safe and feasible and may be applied to all those high rick individuals who are identified to have IPMN during observation and follow-up.

The following work was undertaken during my PhD research and was subsequently published in *Pancreas* as:

Nicholson JA, Greenhalf W, Jackson R, et.al. "Incidence of Post-ERCP Pancreatitis From Direct Pancreatic Juice Collection in Hereditary Pancreatitis and Familial Pancreatic Cancer Before and After the Introduction of Prophylactic Pancreatic Stents and Rectal Diclofenac".[249]

Materials and Methods

Study Design

Participants were recruited from among UK residents on the EUROPAC registry. Written informed consent was obtained for screening by imaging, measurement of serum CA19-9 levels and collection of pancreatic juice by ERCP. Imaging comprised computed tomography (CT) or magnetic resonance imaging (MRI) for both FPC and HP and in addition EUS for FPC. Patients could opt just for imaging without undergoing ERCP. Screening in patients with HP was not commenced before they were aged at least 40 years.[108, 116, 140, 250] Individuals from FPC kindreds were either a first degree relative (sibling, offspring, or parent) or a second degree relative (grandchild, nephew, or niece) of an affected individual. Screening in FPC kindreds was commenced 10 years sooner than the youngest death from pancreatic cancer in that family or from 40 years of age if the youngest death was over 50 years of age.[116, 138] The study is approved by the relevant Research Ethics Committees [LREC, AAGPM97199 (1998); MREC, 07/H1211/96 (2007) & 07/H1008/153 (2007), Protocol Version 3 (2014)]. The study is co-sponsored by the University of Liverpool and The Royal Liverpool and Broadgreen University NHS Hospitals Trust. The characteristics of patients, procedures and complications were regularly reviewed by the EUROPAC Screening Trial Steering Committee.

Pancreas juice collection and analysis

ERCP was undertaken by consultant gastroenterologists in the Endoscopy Unit at the Royal Liverpool University Hospital as a day case. Each patient was given 750mg ciprofloxacin, before and after ERCP. Intravenous sedation consisted of midazolam (1-5mg) with hyoscine butyl-bromide (20-40mg) as an anti-peristaltic agent and either fentanyl (50-100µg) or pethidine (25-50mg). Selective cannulation of the pancreatic duct was confirmed by radiological screening without contrast followed by

administration of 1IU/Kg secretin (Sanochemia, Germany) intravenously. After two minutes, pancreatic juice was collected by gentle aspiration from the pancreatic catheter. Pancreatic juice was analysed for *TP53* and *KRAS* mutation and quantification of *CDK2Na* promoter methylation.[126]

Post-ERCP acute pancreatitis

This was defined as an increase in serum amylase of at least three times (>450 iu/L) the upper limit of normal (150 iu/L) and abdominal pain within 48 hours of ERCP. In HP patients there was no requirement for a raise in the serum amylase. Duration of inpatient stay was defined as the number of days spent in hospital from the day of the ERCP procedure and severity was determined by the Atlanta criteria.[251] All complications were reported to the relevant authorities (Sponsor and Research Ethics Committees) as serious adverse events in line with Good Clinical Practice.

Prophylaxis measures

A 3cm 5Fr self-expelling stent (single pig-tail, no flap; Cook Medical, Zimmon) was deployed using a 5Fr introducer following aspiration of pancreatic juice. Confirmation of stent placement was by observation of the pigtail end of the stent protruding into the duodenum via the ampulla of Vater. Additionally, diclofenac (50mg) was given per rectum within 30 minutes of the procedure. Patients were routinely discharged the same day if there were no complications. For all patients who had had a stent inserted a plain abdominal X-ray was undertaken 6 weeks after the ERCP to determine whether the pancreatic stent had been expelled. All patients were contacted at 6 weeks following the ERCP with the results of their molecular analysis and any other post-ERCP complications were identified and recorded.

Statistical analysis

Continuous data are presented as median and inter-quartile intervals (IQR), categorical data are displayed as tables of counts and associated percentages. Associations of factors across patient groups were carried out using a two tailed Mann-Whitney U test for continuous data and Fisher's exact test for categorical variables. Risks are presented as odds ratio with associated 95% confidence intervals and are obtained from the parameters of univariate logistic regression models. Multivariate

modelling was not attempted due to the small number of events. All analyses were carried out using R version 2.15 (R Foundation for Statistical Computing, Vienna, Austria). P values are assessed at the 0.05 level throughout.

Results

The first patient was enrolled on the 6th of January 1999 and the study censored on the 1st December 2013 for this analysis. There were 211 individuals, 187 individuals from FPC kindreds and 24 patients with HP. There were 80 pancreatic juice samples collected from 60 of these individuals; the remaining 51 individuals only underwent imaging. (CONSORT diagram, Figure 13).

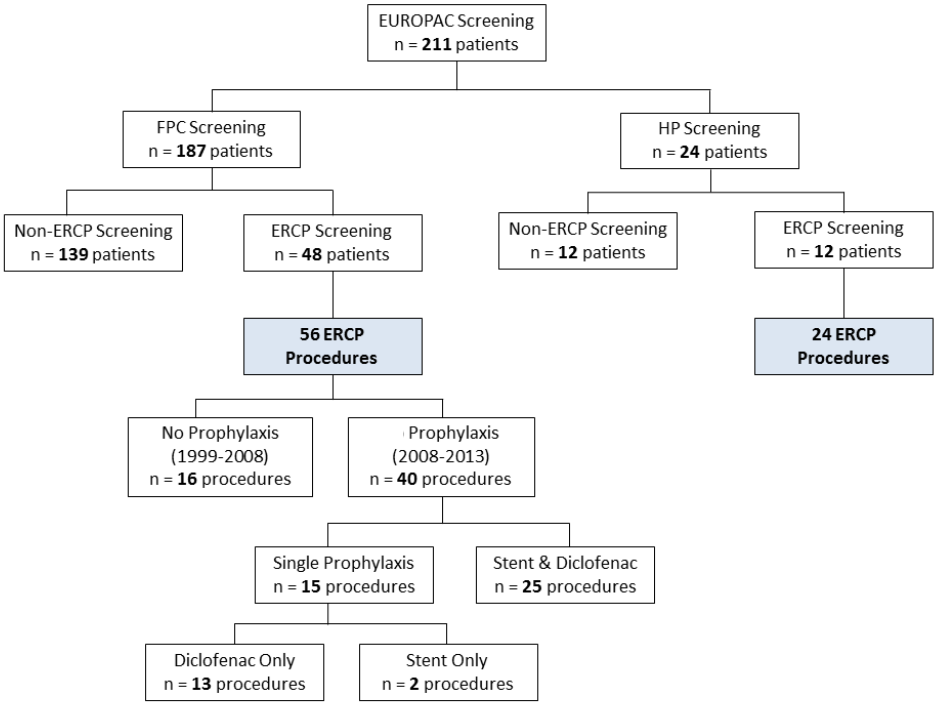


Figure 13: CONSORT diagram for sub-groups of individuals undergoing screening using ERCP.

Fifty-six of the ERCP procedures were in individuals from FPC kindred and 24 in HP patients. In the FPC cohort one individual underwent three ERCPs and five individuals underwent two ERCP procedures. In The HP cohort one patient underwent six ERCPs, one patient underwent five ERCPS and three underwent two ERCP procedures. The demographic details of all patients are presented in Table and the genetic background in patients associated with pancreas cancer syndromes and inherited pancreatitis is shown in **Error! Reference source not found.**17.

Table 16: Demographic data for individuals undergoing screening with ERCP.

Variable		Total	Screening Group		p
			FPC	HP	
		N = 60	N = 48	N = 12	-
Age (years)	Median (IQR)	54 (45.25-62)	53 (44.25-60)	61 (47.25-62)	0.216
Gender	Male	27 (45%)	22 (46%)	5 (42%)	1.0000
	Female	33 (55%)	26 (54%)	7 (58%)	
Body Mass Index (Kg/m^2)	Median (IQR)	26.3 (24.4-31)	27.95 (25-32.32) Missing in 6	23.7 (22.6-24.6) Missing in 5	**0.004**
Smoking	Never Smoked	33 (55%)	24 (50%)	9 (75%)	0.1945
	Previous/Current Smoker	27 (45%)	24 (50%)	3 (25%)	
Alcohol (units/week)	Median (IQR)	5 (1-15)	7 (2-15.5) Missing in 3	0 (0-1)	**<0.001**
Diabetes Mellitus	No Yes	53 (88%) 7 (12%)	47 (98%) 1 (2%)	6 (50%) 6 (50%)	**0.0001**

Table 17: Genetic background of patients with pancreatic cancer syndromes and inherited pancreatitis.

Inherited Syndrome	Syndrome variant or Mutation	Individuals N = 60
Familial Pancreatic Cancer Syndrome[33, 124, 252]	Familial Pancreatic Cancer with unknown causative mutation (FPC)	41
	Hereditary Non-Polyposis Colonic Carcinoma; Lynch Syndrome (HNPCC)	2
	Peutz-Jeghers Syndrome (PJS)	2
	Familial Atypical Multiple Malignant Melanoma (FAMMM)	2
	Breast Cancer Type 2 (BRCA2)	1
Hereditary Pancreatitis[138, 140, 250]	Cationic Trypsinogen 1(*PRSS1*), p.R122H	5
	Cationic Trypsinogen 1(*PRSS1*), p.N29I	5
Familial Idiopathic Pancreatitis[253]	Serine Protease Inhibitor Kazal-Type 1 (*SPINK1*), p.N34S	2

A Trial Steering Committee review was undertaken in August 2008 after 34 ERCPs had been performed. There were seven (21%) cases of PEP, all seven in the FPC cohort which had had 16 ERCPs versus no cases of PEP in the HP cohort which had had 18 ERCPs (p= 0.0021). The study was suspended until the Committee completed a review of existing procedures. Changes were incorporated into Protocol version 2 (21st November 2008) and the study was recommenced on 28th November 2008. The new procedure now routinely employed soft-tipped wire insertion into the pancreatic duct and

prophylaxis with self-expelling 5-French plastic pancreatic stents and rectal diclofenac for FPC individuals.[254, 255] Following the introduction of prophylaxis the incidence of PEP in FPC individuals fell to six (15.0%) of 40 procedures compared to 7 (43.8%) of 16 procedures without prophylaxis (p=0.0347). Again there was no PEP in the six further ERCP procedures in the HP cohort performed after 2008 (Figure 14).

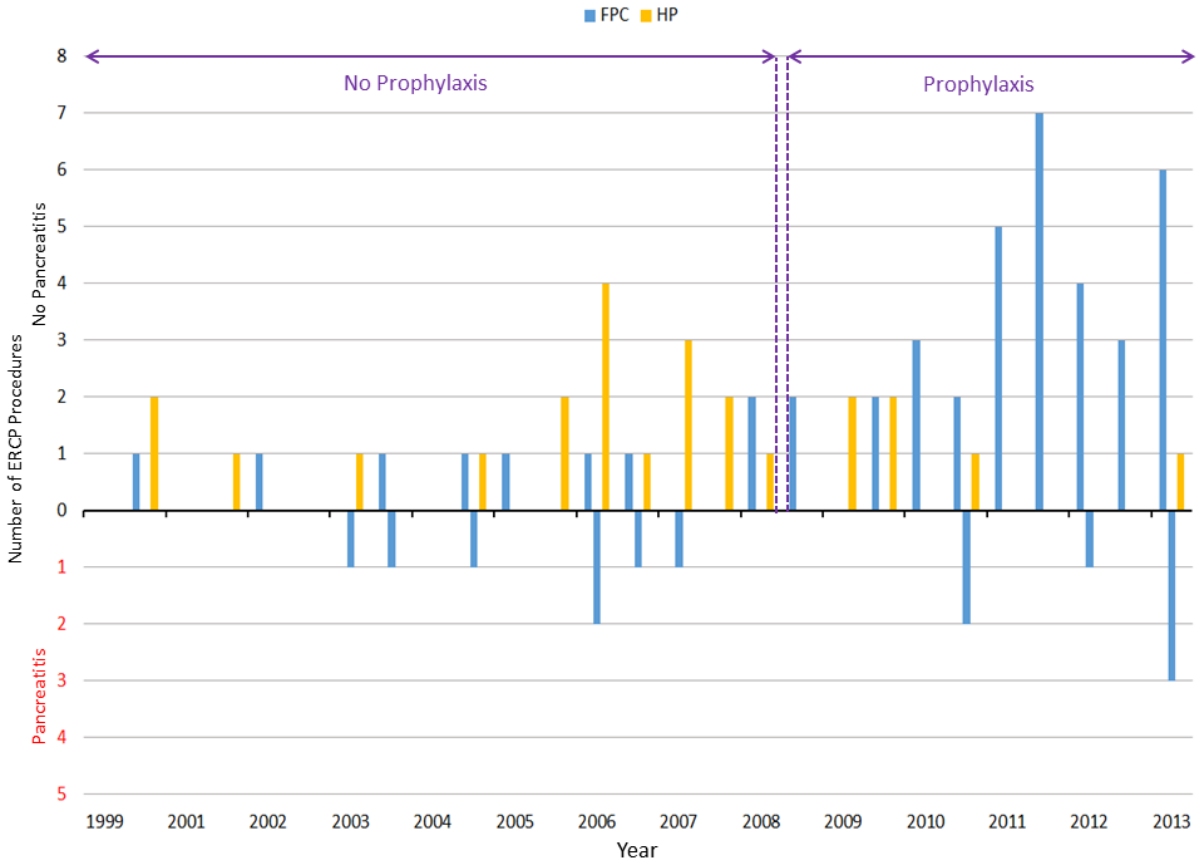


Figure 14: A Time-Event waterfall plot for incidence of PEP among both cohorts.

Endoscopic stent placement was attempted in all 40 cases of the FPC cohort but this was not possible in 13 (33%) due to tortuosity of the main pancreatic duct. Of these 13 cases without a stent one (7.7%) developed PEP. The deployment of the pancreatic stents was safe and well tolerated. Of the six cases of pancreatitis that had had prophylaxis, four individuals had had both stent and diclofenac, one had had a stent only and one had had diclofenac only. PEP occurred in four out of 25 cases with dual prophylaxis (stent and diclofenac) compared to two out 15 cases with single prophylaxis (stent or diclofenac) (p=0.702). The only factor associated with PEP in FPC individuals, was the use of

prophylactic measures (Table 18). There was one severe case of pancreatitis and this occurred in the non-prophylaxis group; there were no deaths. The median (IQR) length of hospital stay for patients with pancreatitis in the non-prophylaxis and prophylaxis groups was 5 (3-15) and 5 (0-10) days respectively (p=0.628). The only other ERCP related complication was one instance of duodenal perforation managed conservatively. By six weeks 24 (89%) of the stents were spontaneous expelled and the remaining three were removed uneventfully using upper gastrointestinal duodenoscopy. There were no instances of blocked stent or pathological migration.

Table 18: Analysis of post-ERCP pancreatitis following 56 ERCP procedures performed in individuals from FPC kindreds.

Factor		ERCP procedures n = 56	Post-ERCP pancreatitis n = 13	Odds Ratio (95% CI)	P-value[b]
Age (years)[a], Median (IQR)		53 (44.75-60)	50 (42-54)	0.97 (0.92, 1.02)	0.1113
Gender	Female	30	9 (30%)	0.43 (0.08, 1.84)	0.2238
	Male	26	4 (18%)		
Body Mass Index (Kg/m2)[b], Median (IQR)		26.9 (24.73, 31.35)[d]	29.8 (25.6, 35.6)	1.06 (0.99, 1.13)	0.0585
Smoking	Never Smoked	28	8 (29%)	0.55 (0.12, 2.28)	0.5279
	Previous/Current Smoker	28	5 (18%)		
Alcohol intake units/week Median (IQR)[c]		5(1 - 15)[e]	7 (2 -20)	1.08 (0.98, 1.21)	0.5338
Prophylaxis	No	16	7 (44%)	0.23 (0.06, 0.84)	**0.035**
	Yes	40	6 (15%)		

[a]Odds Ratio given for likelihood of developing post-ERCP pancreatitis if age is >median value.
[b]Odds Ratio given for likelihood of developing post-ERCP pancreatitis if BMI is >median value.
[c]Odds Ratio given for likelihood of developing post-ERCP pancreatitis if alcohol >median value.
[d]Missing data in 6 cases.
[e]Missing data in 3 cases.

In summary during the whole period (1999-2013), PEP occurred in 13 (16.3%) instances following ERCP, all of which occurred following the 56 ERCPs in the FPC cohort (23.2%) and none in the HP cohort (p=0.0077).

[b] *P-values presented are obtained from two-tailed Fisher or Mann Whitney U tests and are in general agreement with P-values obtained via Wald tests.*

Screening outcomes

Two patients with HP with positive pancreatic juice molecular tests had total pancreatectomy. The CT images did not show the presence of any malignant lesion. Both patients already had exocrine failure and also endocrine failure requiring insulin. The first patient had mutant *KRAS* (p.G12V), mutant p53 (p.G245D) and *CDKN2a* promoter methylation >50%. The second patient had mutant *KRAS* (p.G12R), wild-type *TP53* and *CDKN2a* promoter methylation of 17.6%. In both cases histological examination of the resected pancreata revealed pancreatic intraepithelial neoplasia grades 1A, 1B and 2.

Molecular analysis of pancreatic juice of one individual in the FPC cohort with a strong family history of pancreatic cancer (father, paternal uncle and two brothers all with pancreatic cancer) identified wild-type *KRAS*, mutant p53 (p.V225fs), and *CDK2Na* promoter methylation of 41.7%. The pancreas-specific CT did not shown any evidence of a tumour but EUS revealed a hypoechoic focus in the body of the pancreas. EUS fine-needle aspiration was undertaken and cytology revealed nuclear atypia. Blinded molecular analysis of the cytology tissue revealed the same p53 mutation (p.V225fs). Repeat EUS six weeks later however revealed normal imaging and fine-needle aspiration of the same area did not identify any abnormal cells on cytology. Pancreatic juice molecular analysis was repeated 12 months after the initial ERCP and this now identified wild-type *KRAS*, wild type *TP53* and normal levels of *CDK2Na* promoter methylation (<12%). Subsequent surveillance EUS examinations were normal. The individual remains under active follow-up 34 months after the original pancreas juice collection.

Another individual from the FPC cohort had wild-type *KRAS*, mutant *TP53* (p.L188P) and normal *CDK2Na* promoter methylation of 3.47%. CT and EUS imaging were normal however and the individual remained under active follow-up 14 months after the original pancreas juice collection.

Discussion

Patient-related factors associated with a higher risk of PEP have commonly included age of 18-35 years, a history of recurrent acute pancreatitis, a previous episode of PEP, and sphincter of Oddi dysfunction.[255-258] This study has found that the risk of PEP in patients with HP undergoing screening by direct pancreatic cannulation for pancreatic juice collection following injection of secretin was very

low (no events in 23 procedures). Two previous studies of interventional ERCP in patients with severe symptoms from HP have also suggested a low risk of PEP, but our study is the only one focussed on direct pancreatic duct cannulation and juice collection.[259, 260] In marked contrast to the risk of PEP in patients with HP, the risk to patients with FPC was very high and this was much greater than might have been previously anticipated.[254, 261, 262]

The difference in the risk of PEP between individuals from FPC kindreds and HP patients could not be explained by differences in age, gender, body mass index or smoking status. Individuals from FPC kindreds have normal sized ducts, which therefore would have predicted that this group would be at higher risk of PEP than those with a diseased pancreas. Patients with HP are much more likely to have diabetes mellitus as part of the disease.[108, 250] FPC individuals had higher alcohol consumption than HP patients but this was not shown to be a contributory factor; patients with HP are likely to drink less alcohol because of medical advice from a young age. The very low risk of PEP in patients with HP may be due to the fact that they are more likely to have dilated ducts along with marked pancreatic parenchymal fibrosis and atrophy and hence have less acinar tissue being exposed to trigger factors.[250, 263]

Direct cannulation of a normal main pancreatic duct and aspiration of pancreatic juice following intravenous injection of secretin is the most likely cause of the PEP.[264] Whilst the use of intravenous injection of secretin during ERCP in our study may have contributed to the high incidence of pancreatitis in the FPC group, a randomized double-blind controlled trial of intravenous secretin given immediately before ERCP has previously been shown to reduce the frequency of PEP from 15.1% in the placebo group to 8.7% in the secretin group[265]

A significant reduction in PEP using either a self-expelling pancreatic stent or diclofenac has been confirmed by several recent pivotal studies and meta-analyses.[266-269] In the current study the incidence of PEP in individuals from FPC kindreds was significantly reduced by using prophylaxis methods but the incidence is still too high in this group of patients to recommend continued use. Instead the EUROPAC Protocol has now adopted duodenal sampling following secretin injection to

determine molecular changes.[236] It still needs to be established whether duodenal samples have loss of diagnostic sensitivity from (1) the effects of dilution, (2) interference by duodenal derived DNA and (3) contamination by gut flora. Because of this relative uncertainty and the minimal risk in HP patients the EUROPAC screening protocol continues to employ direct pancreatic duct cannulation and aspiration for pancreatic juice in this particular group.

The clinical significance of molecular changes in pancreatic juice and duodenal samples needs to be further established. It is already known that *KRAS* mutations are often found in pancreatic juice from controls as well as those with chronic pancreatitis pancreatic cancer.[139, 270] This study has also found one example of reversion of mutant to wild type *TP53* and high *CDK2Na* promoter methylation reduction to normal levels with repeat sampling on observation. Despite the high theoretical risk of pancreatic cancer in FPC kindreds, the yield from current screening programmes is low. An analysis of 988 individuals from FPC kindreds undergoing screening identified only 25 (2.5%) patients who had resection: due to high-grade dysplasia in 23 and early pancreatic cancer in the remaining two.[271] The improvements in protocols from the EUROPAC and other screening programmes should encourage a higher participation by clinicians and patients at high risk from pancreatic cancer.

5.4 – Demographics of Heidelberg Samples

Seventy two tissue samples were obtained from The University of Heidelberg, all were samples of cystic neoplasms from resected pancreata obtained between 2000 – 2005. These samples were received frozen and subsequently prepared as described in Chapter 4. Forty two samples were randomly selected to be used in ion torrent analysis. The number was based on 20 ion torrent chips with 4 chips to be utilised in experimental design. Demographic data are therefore presented for the total cohort of samples (n=72) and the ion torrent subset for which mutational data were obtained (n=41, one sample did not yield sufficient DNA for ion torrent analysis). Below are presented a CONSORT diagram and table comparing the Ion Torrent Cohort to the Total Sample (PB = pancreatobiliary).

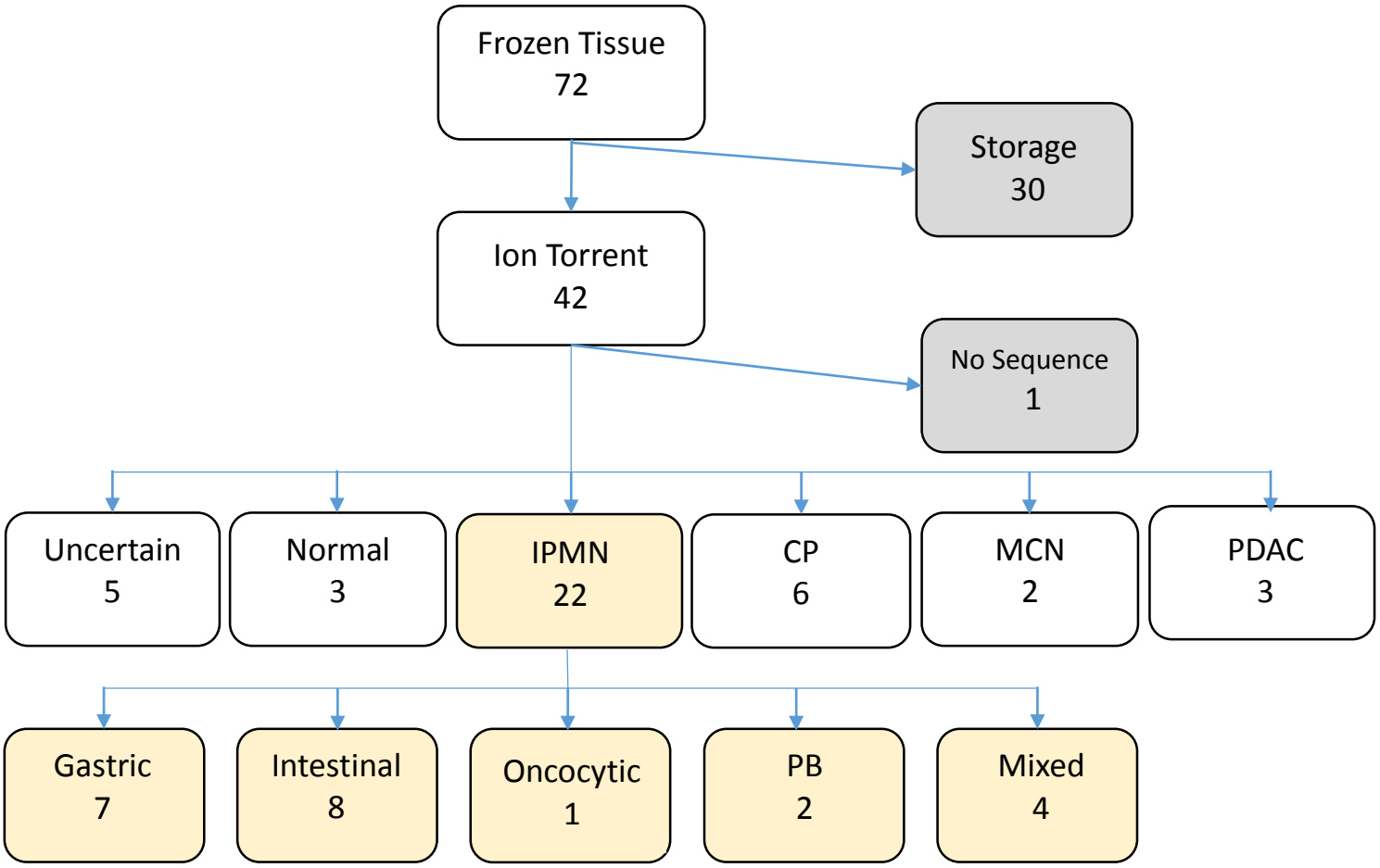


Figure 15: CONSORT diagram of Ion Torrent samples, PB = pancreatobiliary, MCN = mucinous cystic neoplasm.

Table 19: Demographics of Ion Torrent cohort vs. total sample.

Variable	Total Sample	Ion Torrent Cohort
Median Age (years)	63.35 (IQR: 53.18 – 69.25) n=72	65.50 (IQR: 61.15 – 71.30) n=41
Median Survival (months)	33.48 (IQR: 18.07 – 50.76) n=56	36.57 (IQR: 19.40 – 54.10) n=35
Gender (M:F)	36:36 n=72	25:16 n=41
Deaths	12 (16.7%)[a] n=72	10 (24.4%)[b] n=41
HEIDELBERG HISTOLOGY	n=72	n=41
Cystadenoma	14 (19.4%)	5 (12.2%)
IPMN	27 (37.5%)	7 (17.1%)
IPMC	27 (37.5%)	26 (63.4%)
NET	1 (1.4%)	0
PDAC	3 (4.2%)	3 (7.3%)
LOCATION OF TUMOUR	n=70	n=40
Body	9 (12.5%)	5 (12.2%)
Head	34 (47.2%)	20 (48.8%)
Tail	17 (23.6%)	10 (24.4%)
Whole Pancreas	10 (13.9%)	5 (12.2%)
SMOKING STATUS	n=72	n=41
Former	10 (13.8%)	7 (17.1%)
Never	52 (72.2%)	27 (65.9%)
Current	10 (13.8%)	7 (17.1%)
ALCOHOL	n=71	n=41
Daily	14 (19.4%)	8 (19.5%)
Former	1 (1.4%)	1 (2.4%)
Never	25 (34.7%)	16 (39.0%)
Occasional	31 (43.1%)	15 (36.9%)
DIABETES	n=72	n=41
No	59 (81.9%)	30 (73.2%)
Diet controlled	3 (4.2%)	3 (7.3%)
Insulin Controlled	5 (6.9%)	4 (9.8%)
Tablet	5 (6.9%)	4 (9.8%)
WEIGHT LOSS	n=71	n=41
Yes	28 (38.9%)	19 (46.3%)
No	43 (59.7%)	22 (53.7%)

[a]3 deaths were post-operative

[b]1 death was post-operative

Age

This is defined as the age of the patient at the time of resection, in years.

Survial

The number of whole months following resection for which data is available.

Gender

Defined as male or female.

Death

A simple 'yes' or 'no' if the patient is known to have survived up to 5yrs following resection. If the patient is lost to follow-up they are presumed to be alive for the purposes of statistical analyses.

Histology

Can be defined as 'Heidelberg Histology' which is the definitive tissue diagnosis based upon formal assessment of the resected specimen *in toto*; or 'Liverpool Histology' which is the working histological diagnosis based upon independent blinded assessment of a small fragment of the resected specimen by a single pathologist in Liverpool. Described as cystadenoma, intraductal papillary mucinous neoplasm (IPMN), intraductal papillary mucinous neoplasm containing cancer (IPMC), neuroendocrine tumour (NET) or pancreatic ductal adenocarcinoma (PDAC).

Stage of Tumour

Given in the format Ta Nb Mc where:

T = Tumour invasion and 'a' is presented as a number from 0-3 (0 is also known as *tis*).

N = Nodal status and 'b' is given as 0 (no nodes involved) or 1 (lymph nodes contain cancer).

M = Metastatic spread and 'c' is given as 0 (no identified metastases) or 1 (distant metastases present).

Thus an early cancer may be $T_1N_0M_0$ and an advanced cancer with metastases may be $T_3N_1M_1$. See Appendix D for the WHO Classification for Histological Grading in Pancreatic Cancer (6^{th} Edition).

Location of Tumour

This is the anatomical site of the resected tumour within the pancreas as taken from the pathological description of the specimen provided by Heidelberg. It was described as head, body, tail or whole pancreas.

Smoking Status

Self-reported status of the patient in terms of tobacco use, described as either: current smoker; former smoker (stopped >12 months previously) or; never smoked. Quantity and duration of smoking history data were not available.

Alcohol

Self-reported status of the patient in terms of alcohol consumption, described as: drinking >1 unit daily; >1 unit occasionally; former drinker of >1 unit occasionally (having stopped >12 months previously) or; life-long abstinence (1 unit or less and only occasionally).

Diabetes mellitus

Recorded as the presence or absence of a diagnosis of diabetes mellitus at the time of resection. This is described in terms of the modality of treatment which the patient was receiving for their diabetes at the time of resection: No diabetes present; diet controlled diabetes; insulin controlled diabetes or; tablet (metformin or other anti-hyperglycaemic agent) controlled diabetes.

Weight Loss

This is defined as the presence or absence of self-reported unintentional weight loss at the time of resection. Numeric values were not available.

5.5 - Tissue Staining Results

As described in Chapter 4 the tissue slides prepared from the Heidelberg tissue first underwent examination after staining with Haematoxylin and Eosin. Over the following pages examples of H&E and then IHC stained tissue will be presented. Confirmation of histological diagnoses was made in conjunction with Professor Fiona Campbell, Consultant Histopathologist, Royal Liverpool & Broadgreen University Hospital.

H&E and IHC

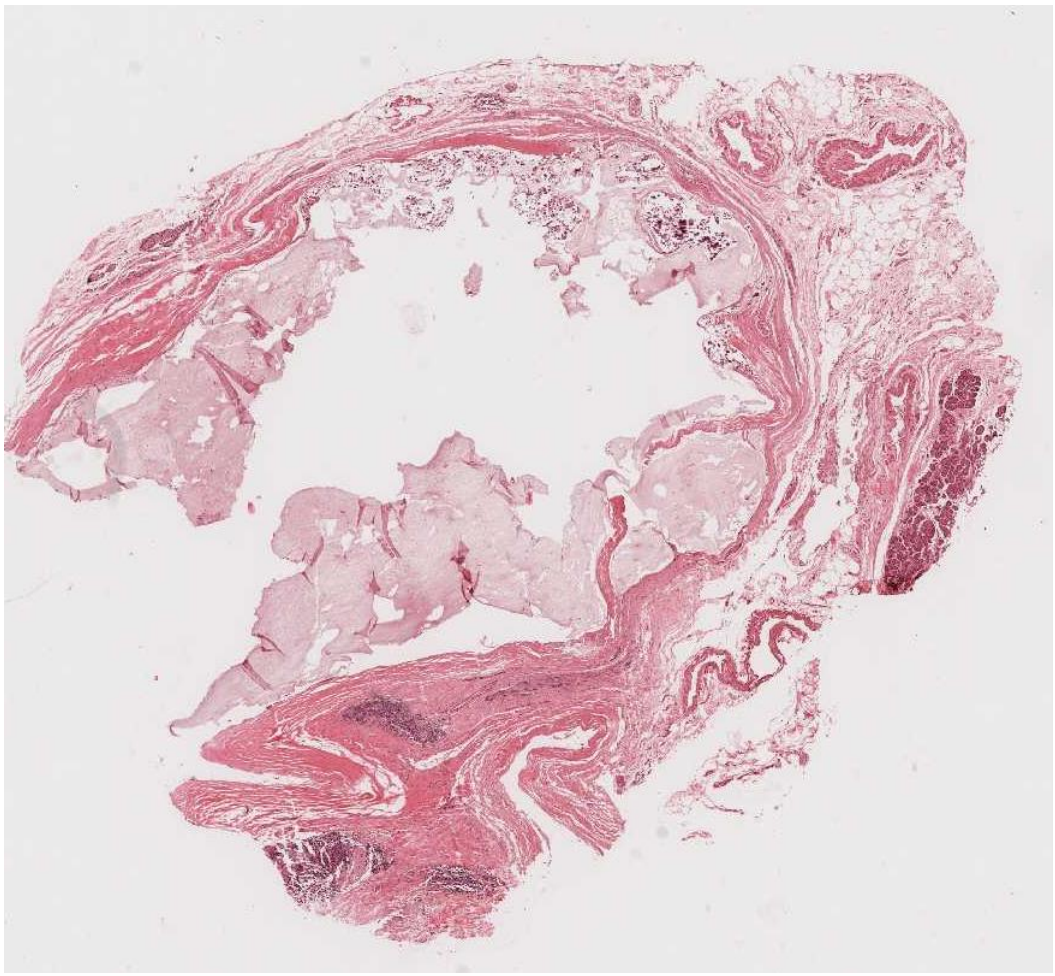


Figure 16: IPMN showing extravasated mucus and calcification, low power H&E (CYST016).

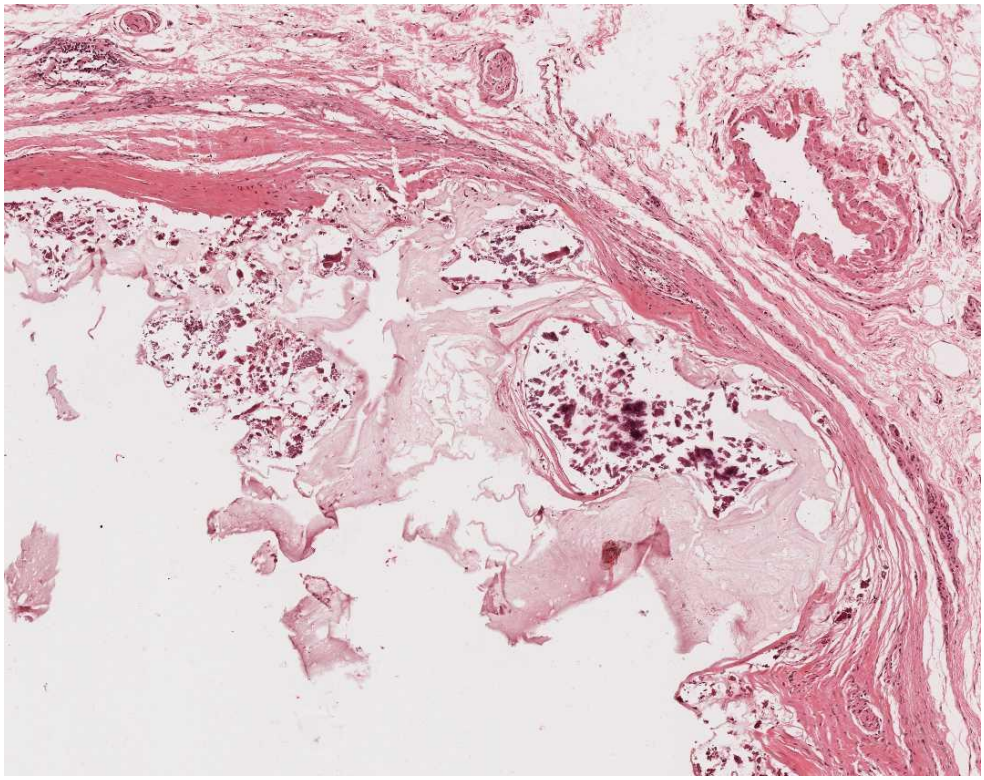


Figure 17: The same image (CYST 016) under medium magnification, H&E.

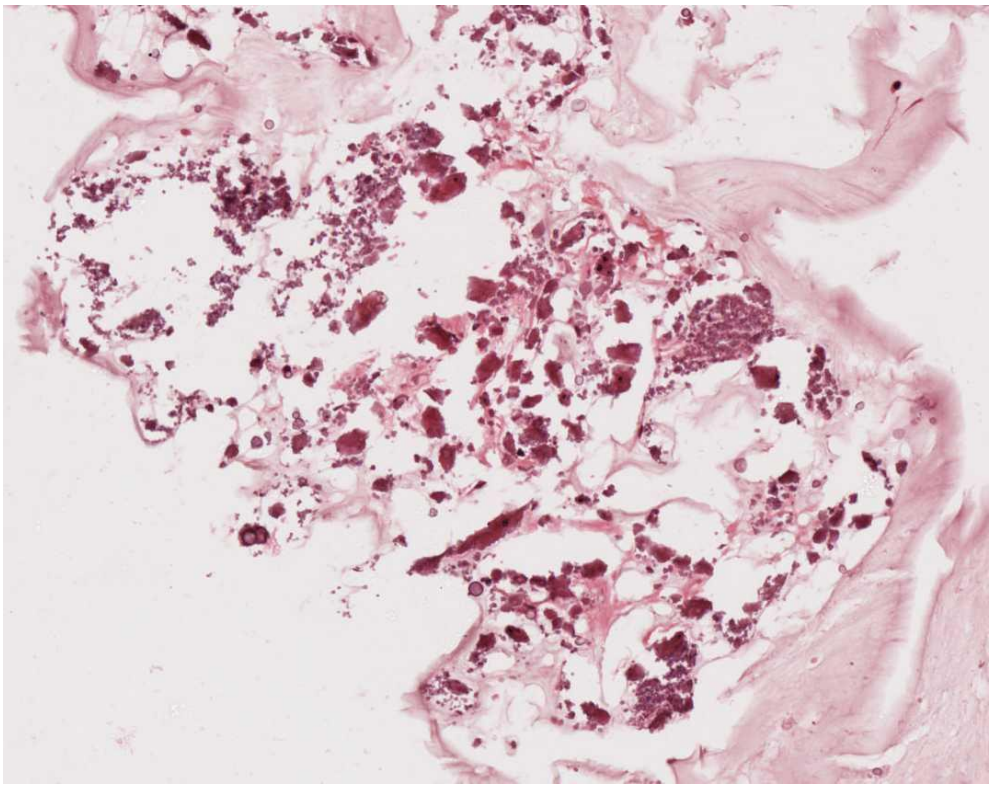


Figure 18: The same image (CYST 016) under high magnification showing scanty epithelial cells, suitable for IHC, H&E.

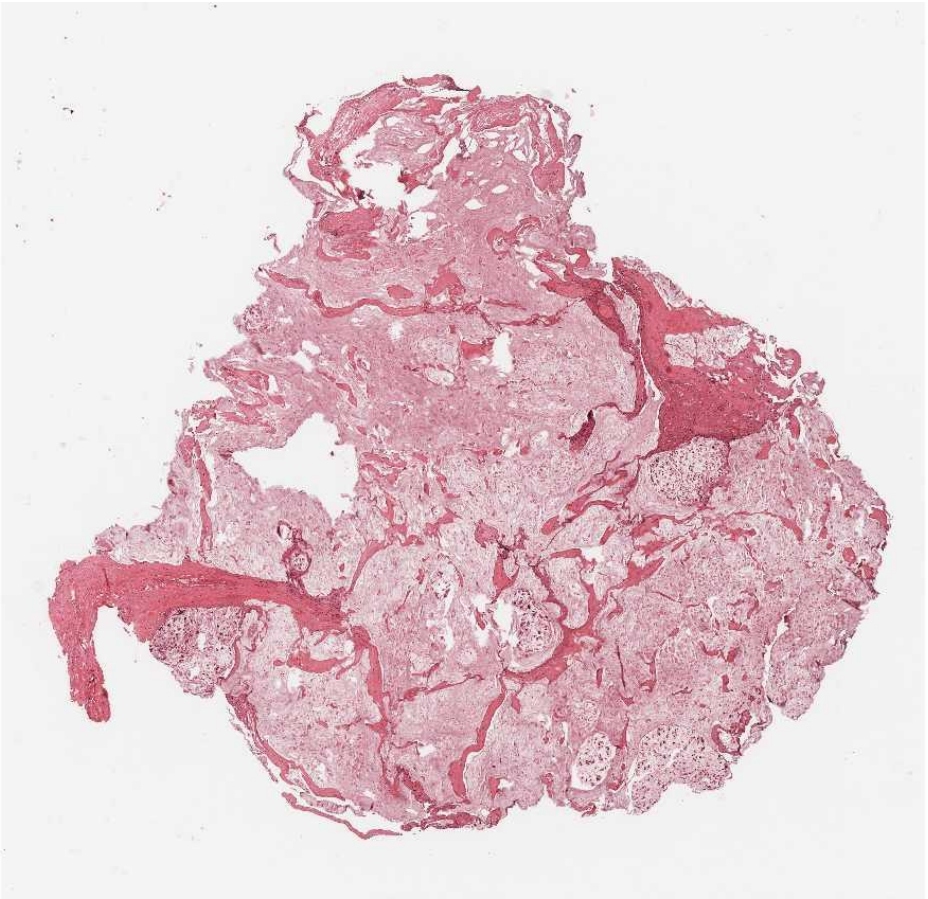


Figure 19: Colloid cancer under low magnification, CYST 014.

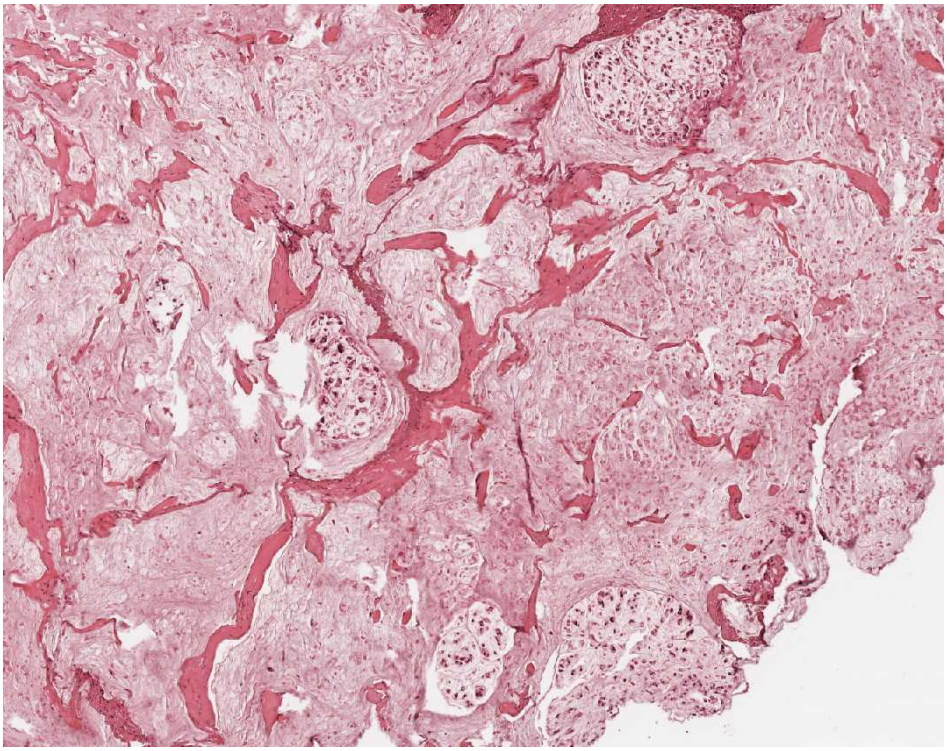


Figure 20: Same image (CYST 014) under medium magnification, H&E.

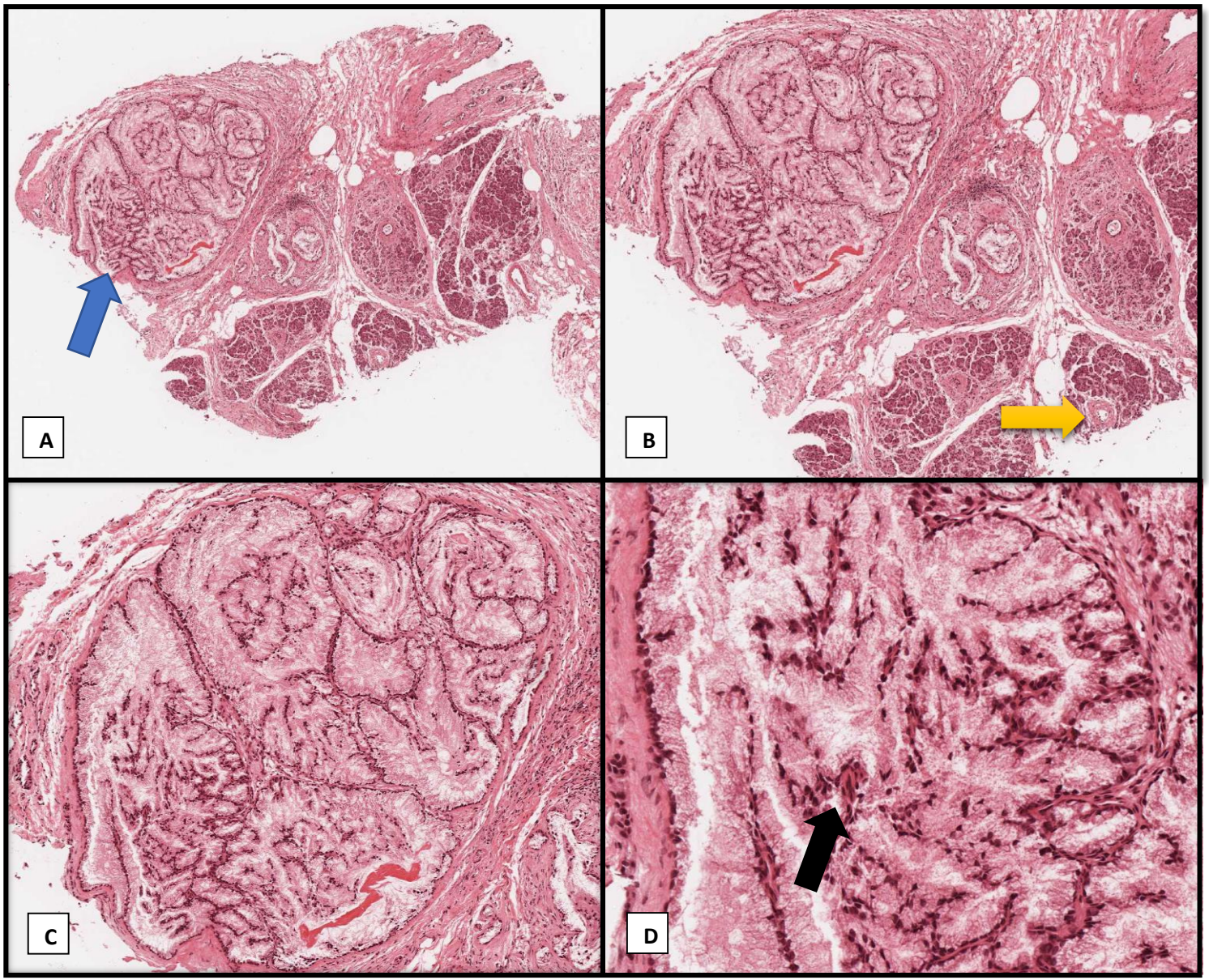


Figure 21: PanIN (black arrow) within IPMN (blue arrow) (CYST 029) under low (A); medium (B); high (C) and highest (D) magnification, H&E. Normal duct arrowed in yellow.

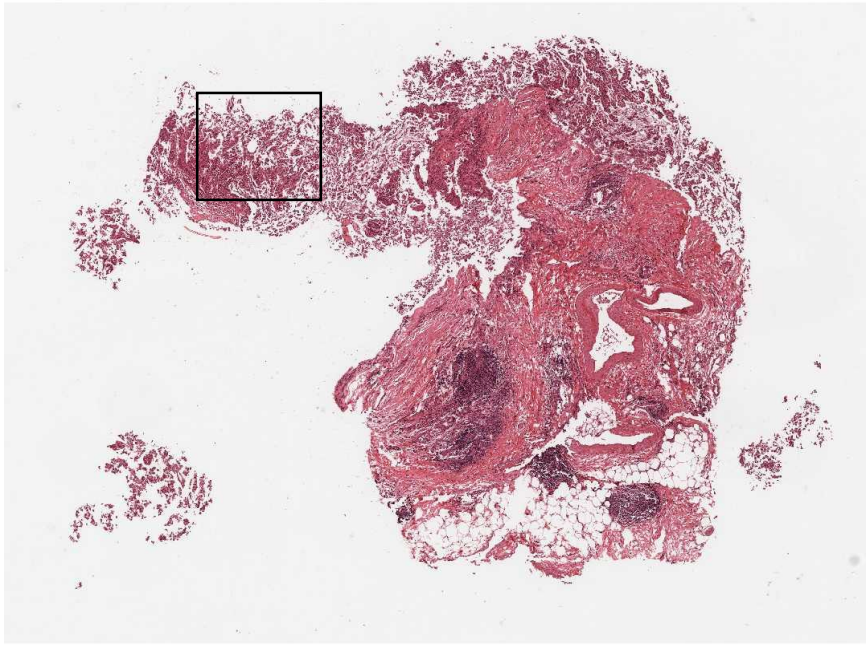


Figure 22: Oncocytic IPMN (CYST 023), low magnification, H&E.

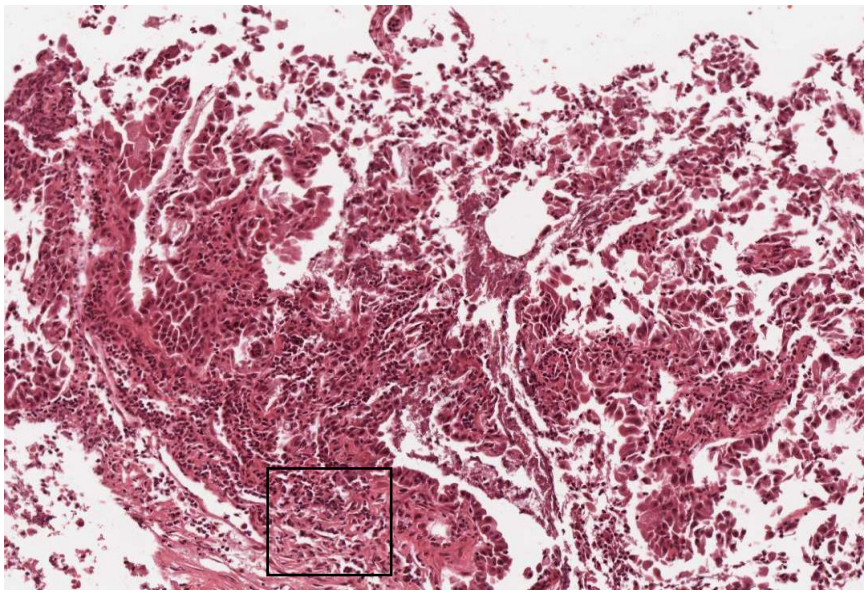


Figure 23: Oncocytic IPMN (CYST 023), medium magnification, H&E.

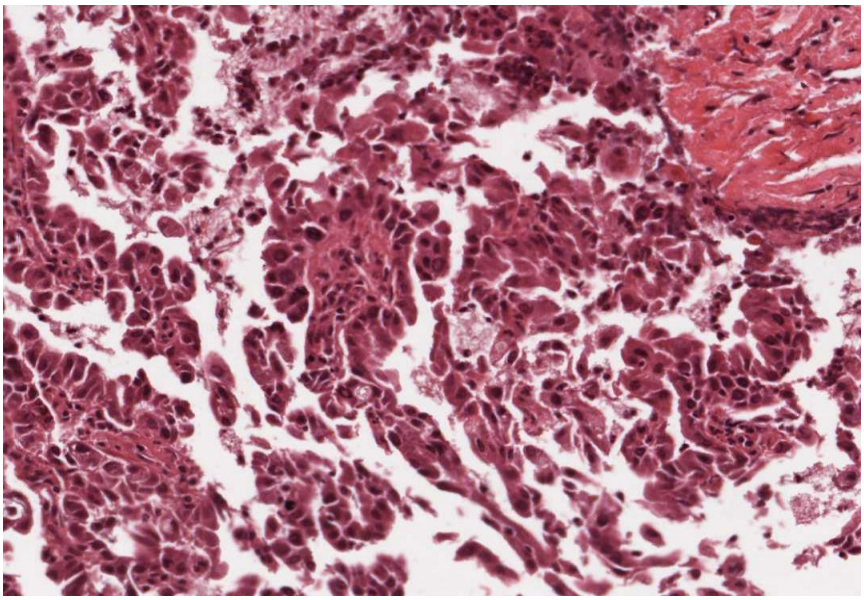


Figure 24: Oncocytic IPMN (CYST 023), high magnification, H&E.

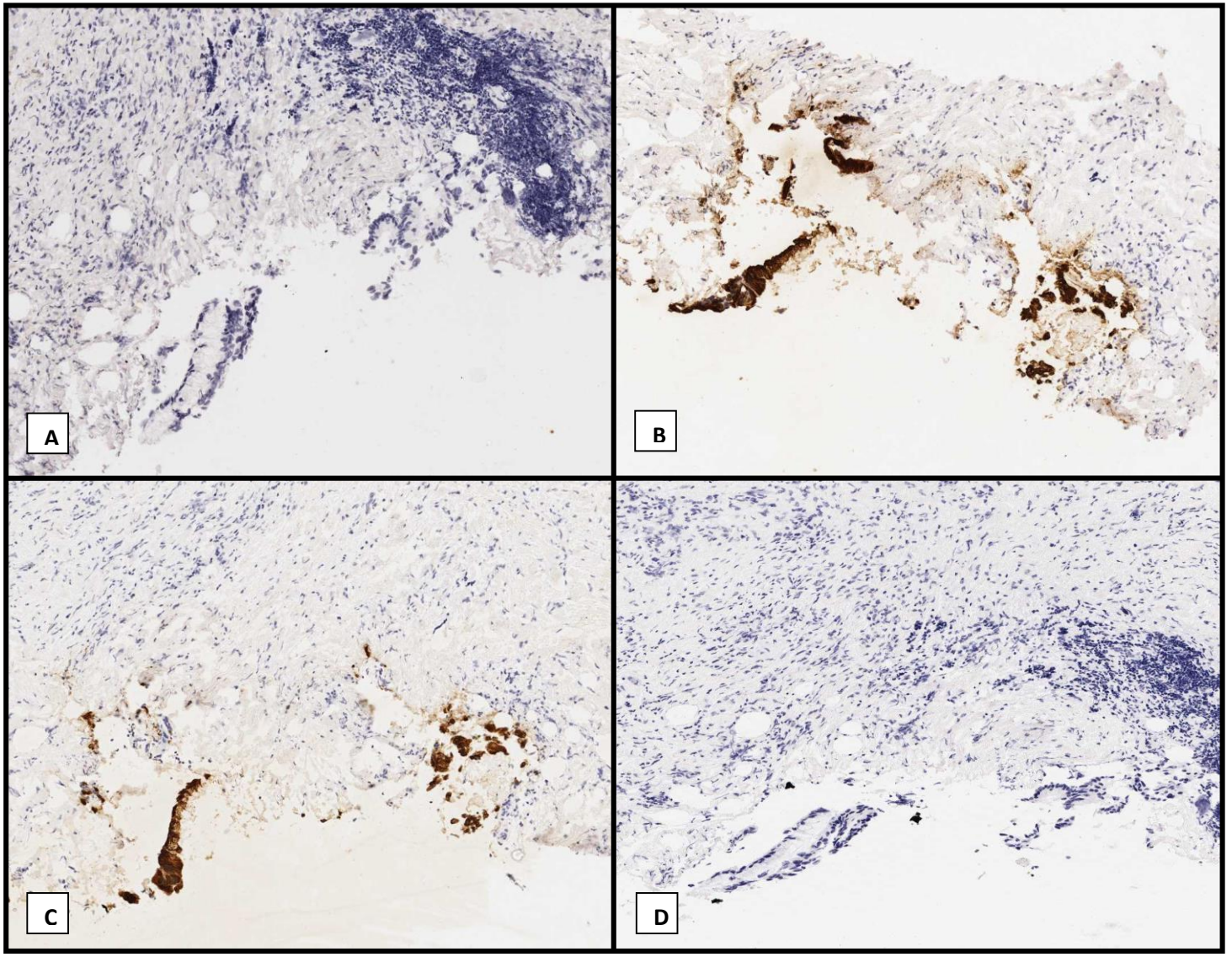


Figure 25: IHC for CYST 010 after MUC 1 (A); MUC 2 (B); MUC 5AC (C) and MUC 6 (D), H&E.

5.6 - Histology

For each of the 41 samples selected, sub-division (a) was sectioned for H&E staining (p.44) and submitted to expert assessment by a single Professor of pancreatic histopathology (Professor Fiona Campbell). This process was performed whilst blinded to the histology reports from Heidelberg.

Table 20 displays the histological assessment made in Liverpool compared to the actual histological result as described by Heidelberg. It must be remembered when comparing these data that the histological diagnosis made in Heidelberg was made in light of the clinical details of each patient from whom the specimens were resected, as well as having the entire resected specimen available for microscopic analysis. In contrast at Liverpool we had a third of a core of representative tissue provided by Heidelberg with no accompanying patient details. Therefore in a number of cases the Liverpool pathologist felt that the tissue sample she had to analyse was insufficient for a diagnosis to be made.

Table 20: Comparison of Liverpool vs. Heidelberg histology.

		Heidelberg Histology			
		IPMN	PDAC	Cystadenoma	TOTAL
Liverpool Histology	IPMN	19	1	2	22
	PDAC	2	1	-	3
	MCN	-	-	2	2
	CP	6	-	-	6
	Normal	3	-	-	3
	Uncertain	3	1	1	5
	TOTAL	33	3	5	41

On the basis of the Liverpool histology diagnosis those specimens thought to represent IPMN (n=21) were submitted for IHC analysis (p.46). In addition as control samples 4 further specimens were included from the Ion Torrent group (1 PDAC, 1 CP and 2 MCN) along with 4 samples collected from patients in the Royal Liverpool University Hospital ("RLUH 1-4") for whom a certain diagnosis of IPMN had been made. In accordance with the protocol outlined in Chapter 4 these samples then underwent IHC analysis for MUC 1, MUC 2, MUC 5AC and MUC 6. The stained slides were then reported as

'positive' or 'negative' for each of the antibodies. On the basis of this they were classified by epithelial sub-type using the key shown in Table 21. These samples are displayed in Table 22.

Table 21: Key for determining epithelial subtype of IPMN using MUC stains. The shaded boxes are positive stain.

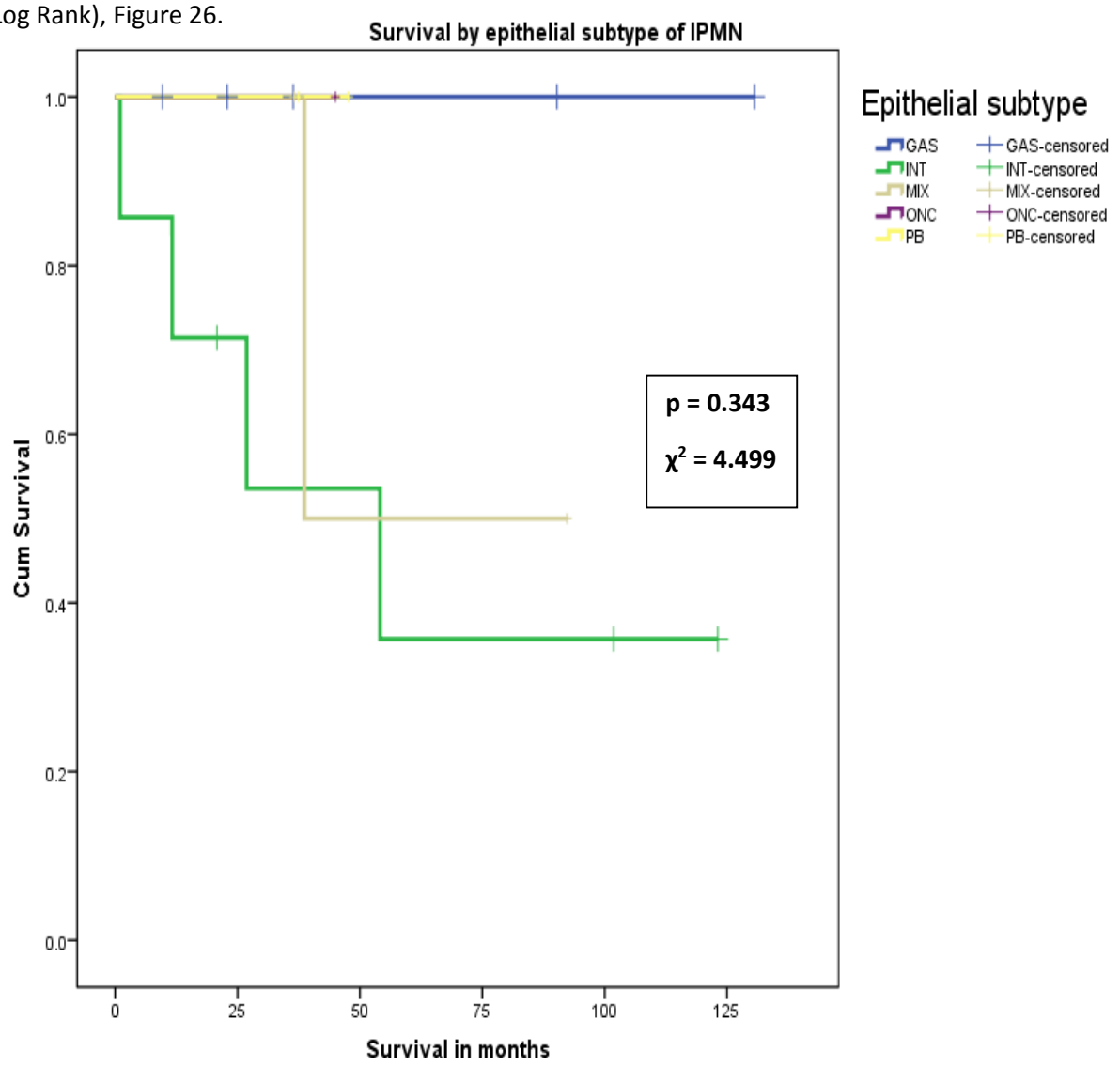
	MUC1	MUC2	MUC5AC	MUC6
Gastric				
Intestinal				
Pancreatobiliary				
Oncocytic				

Table 22: IHC Results for the Ion Torrent cohort.

Sample ID	Histological Diagnosis	Grade of Dysplasia	Epithelial Subtype
CYST001	IPMN	IGD	Gastric
CYST003	IPMN	IGD	Intestinal
CYST004	IPMN	IGD	Intestinal
CYST006	IPMN	LGD	Mixed
CYST009	IPMN	HGD	Mixed
CYST010	IPMN	Colloid	Intestinal
CYST012	IPMN	Colloid	Intestinal
CYST014	IPMN	Colloid	Intestinal
CYST016	IPMN	LGD	Gastric
CYST021	IPMN	Colloid	Intestinal
CYST023	IPMN	HGD	Oncocytic
CYST024	IPMN	Uncertain	Uncertain
CYST029	IPMN	LGD	Gastric
CYST030	IPMN	IGD	Mixed
CYST035	IPMN	LGD	Intestinal
CYST038	IPMN	LGD	Mixed
CYST048	IPMN	IGD	Gastric
CYST054	IPMN	LGD	Gastric
CYST059	IPMN	LGD	Gastric
CYST062	IPMN	LGD	Gastric
CYST073	IPMN	LGD	Pancreatobiliary
CYST002	CP	-	-
CYST017	PDAC	Malignant	Pancreatobiliary
CYST063	MCN	-	-
CYST065	MCN	-	-
RLUH01	IPMN	-	Mixed
RLUH02	IPMN	-	Gastric
RLUH03	IPMN	-	Pancreatobiliary
RLUH04	IPMN	-	Gastric

Epithelial Subtype

As can be seen from Table , 21 samples from Heidelberg had identifiable IPMN epithelial sub-types. Survival data was subsequently found to be available for 18: 5 gastric sub-type; 7 intestinal; 2 pancreatobiliary; 1 oncocytic and 3 mixed. Kaplan-Meier analysis of survival by epithelial subtype amongst the Ion Torrent cohort did not have the power to show any significant difference (p=0.343, Log Rank), Figure 26.



	0	25	50	75	100	125
Gastric	5	3	3	2	1	1
Intestinal	7	4	3	2	2	0
Oncocytic	1	1	1	1	1	1
PB	2	2	1	1	1	1
Mixed	3	3	2	1	0	0

Figure 26: Kaplan-Meier plot to demonstrate survival by epithelial subtype.

Comparison was made between each of the MUC stains and overall survival, data for which was available in 18 cases (85.7%). In addition each of the MUC stains were analysed for correlation with age, gender, tumour status (T_0 – T_3), Nodal Status (N_0 – N_1), presence of metastases (M_0 – M_1), resection margin (R_0 – R_1), primary or recurrent cancer, the location of the tumour within the pancreas, diabetes, smoking, alcohol, weight loss, presence of peritoneal metastases and grade of dysplasia (Low, Moderate, High).

MUC 1

Of 21 samples 7 (33.3%) were found to be MUC1 positive.

No statistically significant difference was established between gender, median age tumour status, nodal status, presence of metastases, resection margin, the location of the tumour within the pancreas, diabetes, smoking, alcohol, weight loss, presence of peritoneal metastases and grade of dysplasia. However, given the small numbers there is insufficient power to conclude no relationships.

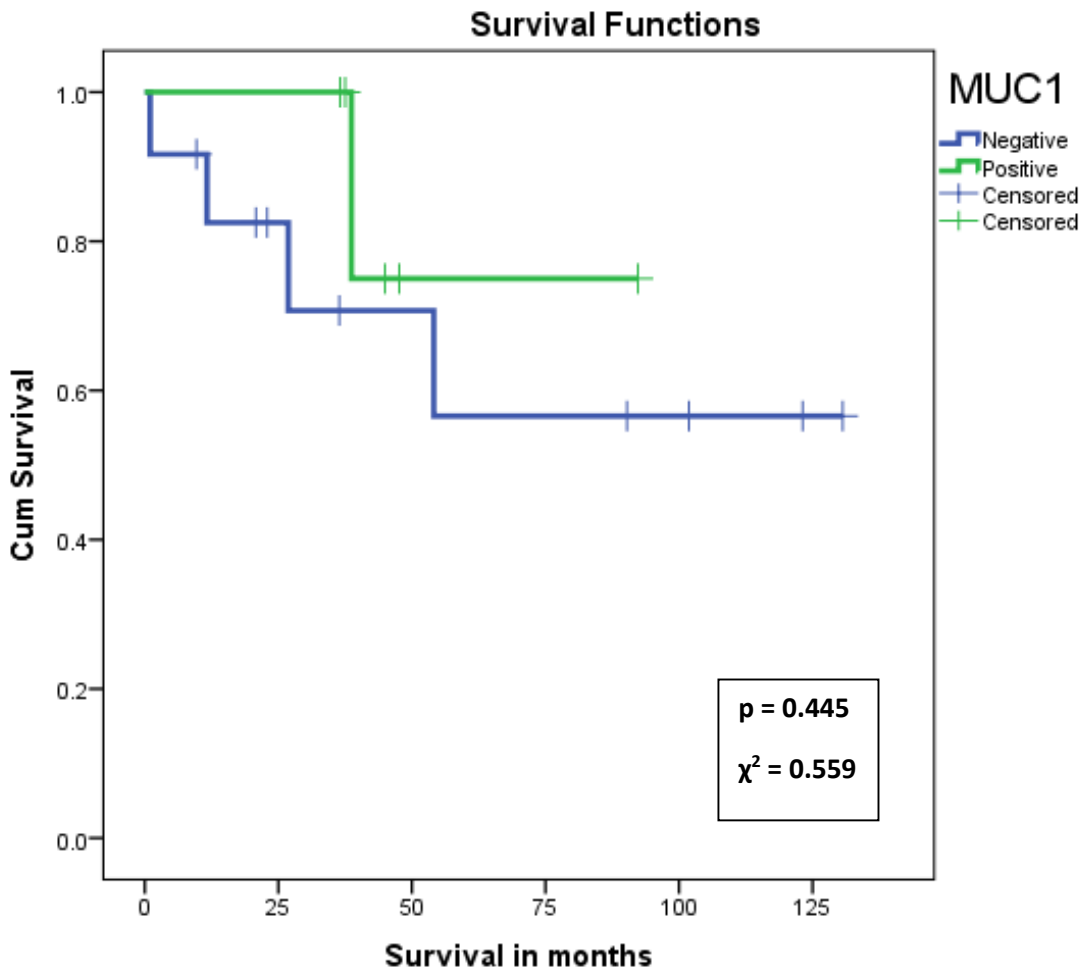
There was a significant likelihood that MUC1 positive samples were associated with recurrent tumours, p=0.030, Table 23.

Table 23: MUC 1 results.

		MUC1		
		Negative	Positive	p.
Gender	Female	7	2	
	Male	7	5	0.32
Tumour Status	Unknown	6	4	
	0	0	1	
	1	2	0	
	2	1	0	
	3	5	3	0.31
Nodal Status	Unknown	6	3	
	0	5	4	
	1	3	0	0.25
Metastases	Unknown	7	3	
	0	6	4	
	1	1	0	0.64
Resection Margin	Unknown	5	3	
	0	9	3	
	1	0	1	0.31

Primary or Secondary Tumour	Unknown	0	1	
	Primary	14	4	
	Recurrence	0	2	**0.03**
Location of Tumour	Unknown	0	1	
	Body	1	2	
	Head	4	2	
	Tail	5	2	
	Whole	4	0	0.23
Diabetes	None	12	5	
	Insulin	1	1	
	Metformin	1	1	0.73
Smoker	Former	3	0	
	No	10	4	
	Yes	1	3	0.09
Alcohol	Daily	4	1	
	Former	1	0	
	No	4	3	
	Occasionally	5	3	0.74
Weight loss	No	8	3	
	Yes	6	4	0.44
Peritoneal metastases	No	13	7	
	Yes	1	0	0.67
Grade of Dysplasia	Cancer	3	1	
	HGD	0	2	
	IGD	3	2	
	LGD	8	2	0.17

There was no significant difference in overall survival between those patients who were MUC1 positive compared to those who were negative, p=0.455 (Log Rank), Figure 27. Survival data were not obtained for 1 MUC 1 positive patient and 2 MUC 1 negative patients.



	0	25	50	75	100	125
Negative	12	6	4	4	3	1
Positive	6	6	1	1	0	0

Figure 27: Kaplan-Meier survival analysis of MUC 1.

MUC 2

Of 21 samples 11 (52.3%) were found to be MUC 2 positive.

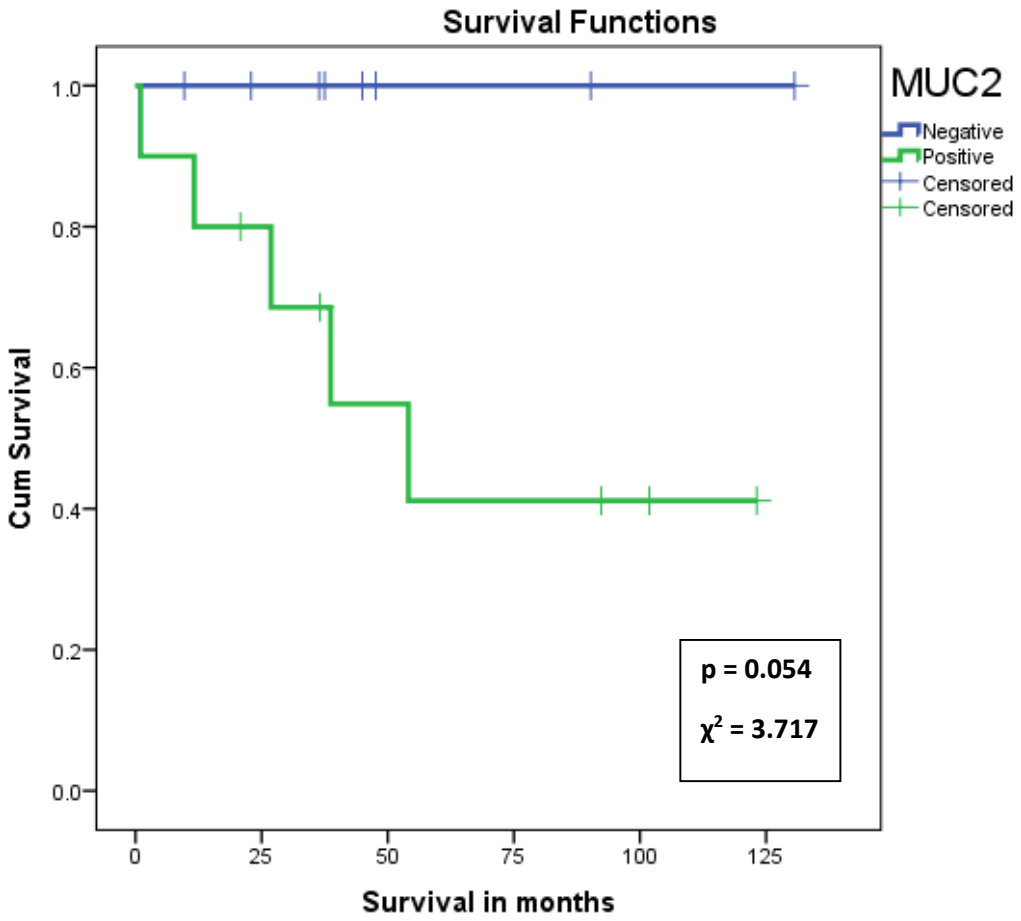
No statistically significant difference was established between median age tumour status, nodal status, presence of metastases, resection margin, primary or recurrent cancer, the location of the tumour within the pancreas, diabetes, smoking, alcohol, weight loss, presence of peritoneal metastases and grade of dysplasia.

There was a significant difference between the number of males and females who were found to be MUC 2 positive, p=0 .024, Table 24.

Table 24: MUC 2 results.

		MUC 2		
		Negative	Positive	p.
Gender	Female	7	2	
	Male	3	9	**0.02**
Tumour Status	Unknown	5	4	
	0	0	1	
	1	1	1	
	2	1	0	
	3	3	5	0.53
Nodal Status	Unknown	5	4	
	0	5	4	
	1	0	3	0.16
Metastases	Unknown	6	4	
	0	4	6	
	1	0	1	0.64
Resection Margin	Unknown	5	3	
	0	4	8	
	1	1	0	0.38
Primary or Secondary Tumour	Unknown	1	0	
	Primary	8	10	
	Recurrence	1	1	0.56
Location of Tumour	Unknown	1	0	
	Body	1	2	
	Head	1	5	
	Tail	4	3	
	Whole	3	1	0.28
Diabetes	Unknown	9	8	
	Insulin	0	2	
	Metformin	1	1	0.36
Smoker	Former	1	2	
	No	8	6	
	Yes	1	3	0.45
Alcohol	Daily	2	3	
	Former	1	0	
	No	5	2	
	Occasionally	2	6	0.22
Weight loss	No	5	6	
	Yes	5	5	0.59
Peritoneal metastases	No	10	10	
	Yes	0	1	0.52
Grade of Dysplasia	Cancer	1	3	
	HGD	1	1	
	IGD	1	4	
	LGD	7	3	0.22

There was borderline significant difference in overall survival between those patients who were MUC 2 positive compared to those who were negative, p=0.054 (Log Rank), Figure 28. Survival data were not obtained for 1 MUC 2 positive patient and 2 MUC 2 negative patients.



	0	25	50	75	100	125
Negative	8	6	2	2	1	0
Positive	10	6	3	3	1	0

Figure 28: Kaplan-Meier survival analysis of MUC 2.

MUC 5AC

Of 21 samples 20 (95.2%) were found to be MUC5AC positive.

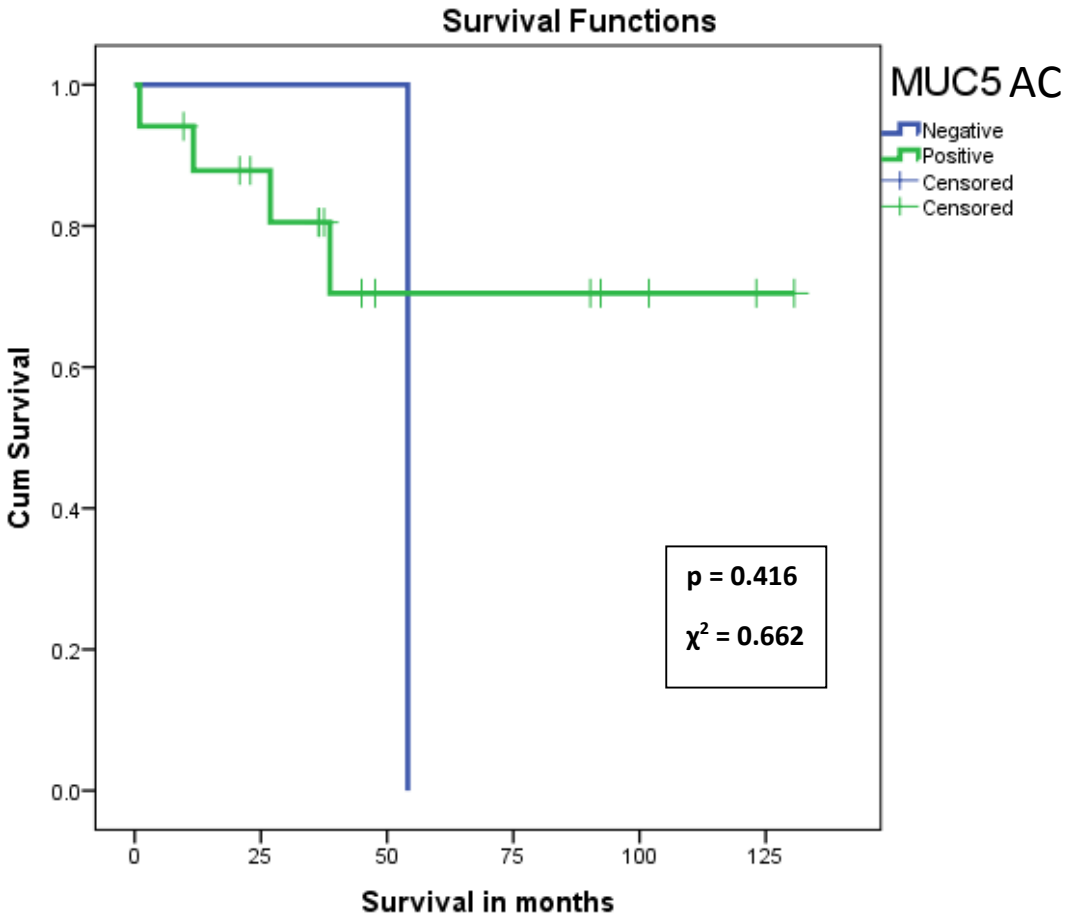
No statistically significant difference was established between gender, median age tumour status, nodal status, presence of metastases, resection margin, primary or recurrent cancer, the location of the tumour within the pancreas, diabetes, smoking, alcohol, weight loss, presence of peritoneal metastases and grade of dysplasia, Table 25.

No significant difference in overall survival could be established when those patients who were MUC5AC positive were compared to those who were negative, p=0.416 (Log Rank), Figure 29. Survival data were unavailable for 3 patients who were MUC 5AC positive.

Table 25: MUC 5AC Results.

		MUC 5AC		
		Negative	Positive	p.
Gender	Female	0	9	
	Male	1	11	0.57
Tumour Status	Unknown	0	9	
	0	0	1	
	1	0	2	
	2	0	1	
	3	1	7	0.91
Nodal Status	Unknown	0	9	
	0	0	9	
	1	1	2	0.25
Metastases	Unknown	0	10	
	0	1	9	
	1	0	1	0.91
Resection Margin	Unknown	0	8	
	0	1	11	
	1	0	1	0.92
Primary or Secondary Tumour	Unknown	0	1	
	Primary	1	17	
	Recurrence	0	2	0.92
Location of Tumour	Unknown	0	1	
	Body	0	3	
	Head	0	6	
	Tail	0	7	
	Whole	1	3	0.35
Diabetes	Unknown	1	16	
	Insulin	0	2	
	Metformin	0	2	0.88
Smoker	Former	0	3	
	No	1	13	
	Yes	0	4	0.77
Alcohol	Daily	0	5	
	Former	0	1	
	No	0	7	
	Occasionally	1	7	0.64

Weight loss	No	0	11	
	Yes	1	9	0.48
Peritoneal metastases	No	1	19	
	Yes	0	1	0.95
Grade of Dysplasia	Cancer	1	3	
	HGD	0	2	
	IGD	0	5	
	LGD	0	10	0.22



	0	25	50	75	100	125
Negative	1	1	1	0	0	0
Positive	17	11	6	6	3	1

Figure 29: Kaplan-Meier survival analysis of MUC 5AC.

MUC6

Of 21 samples 9 (42.9%) were found to be MUC6 positive.

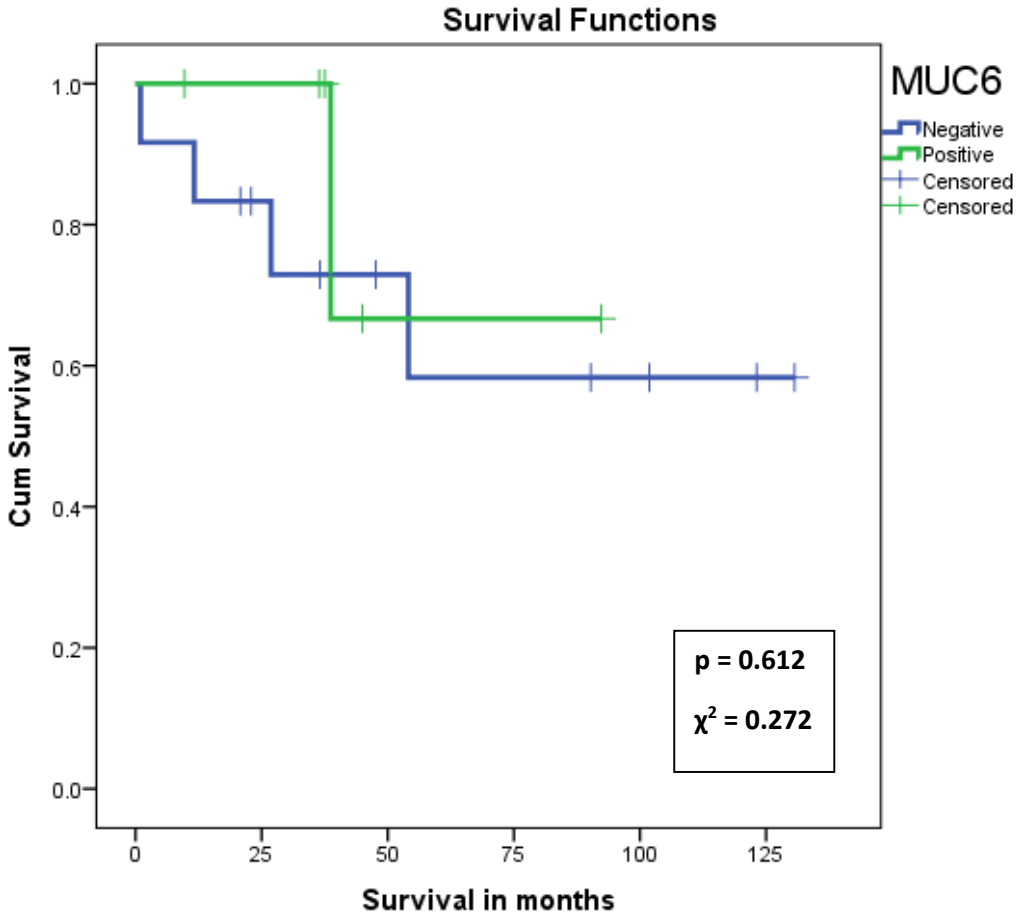
No statistically significant difference could be established between gender, median age tumour status, nodal status, presence of metastases, resection margin, primary or recurrent cancer, the location of the tumour within the pancreas, diabetes, smoking, alcohol, weight loss, and presence of peritoneal metastases. MUC6 did appear to differentiate between the different grades of dysplasia, p = 0.05, Table 26.

Table 26: MUC 6 Results.

		MUC6		
		Negative	Positive	p.
Gender	Female	3	6	
	Male	9	3	0.07
Tumour Status	Unknown	3	6	
	0	1	0	
	1	1	1	
	2	1	0	
	3	6	2	0.72
Nodal Status	Unknown	3	6	
	0	6	3	
	1	3	0	0.38
Metastases	Unknown	4	6	
	0	7	3	
	1	1	0	0.73
Resection Margin	Unknown	0	5	
	0	9	3	
	1	0	1	0.31
Primary or Secondary Tumour	Unknown	0	1	
	Primary	12	6	
	Recurrence	0	2	0.10
Location of Tumour	Unknown	0	1	
	Body	2	1	
	Head	5	1	
	Tail	2	5	
	Whole	3	1	0.20
Diabetes	Unknown	9	8	
	Insulin	1	1	
	Metformin	2	0	0.44
Smoker	Former	3	0	
	No	7	7	
	Yes	2	2	0.27
Alcohol	Daily	4	1	0.46

	Former	1	0	
	No	3	4	
	Occasionally	4	4	
Weight loss	No	7	4	
	Yes	5	5	0.42
Peritoneal metastases	No	11	9	
	Yes	1	0	0.57
Grade of Dysplasia	Cancer	4	0	
	HGD	0	2	
	IGD	4	1	
	LGD	4	6	**0.05**

There was no significant difference in overall survival between those patients who were MUC6 positive compared to those who were negative, p=0.612 (Log Rank), Figure 30.



	0	25	50	75	100	125
Negative	12	7	5	4	3	1
Positive	6	5	1	1	0	0

Figure 30: Kaplan-Meier survival analysis of MUC 6.

Comparison with Mutations

Each of the four MUC stains were analysed for any significant correlation with the presence of mutations which were identified following Ion Torrent analysis. The results of these analyses are given in 5.7 - Ion Torrent Analysis.

5.7 - Ion Torrent Analysis

As described in Chapter 4 the DNA extracted from 41 tissue samples was sequenced for mutations in exons 5, 6, 7 and 8 of *TP53* using the Ion Torrent next generation sequencer. The results are presented here. When comparing the various mutations against MUC stains these results include 21 samples of IPMN previously described AND two samples analysed which were deemed to represent mucinous cystic neoplasm (MCN) histologically in Liverpool, a total of 23.

Data Acquired

Reads were analysed for each of the 10 repeats per sample and mutations were identified if they were present in >9% and <15% or reads at each base. The average fragment length per sample (i.e. median value from 10 repeats) is given in Appendix E along with the average depth of reads of each fragment. The median values for each read of fragment across all 41 samples are given below in Table 27, the median values given are those for all 410 reads.

Table 27: Average depth of reads across every sample analysed using Ion Torrent.

Fragment	Median read Depth
E5F1	6439.5
E5F2	4852.5
E5F3	6823.5
E6	254.5
E7F1	4117.25
E7F2	5445.75
E8F1	3134.25
E8F2	478.5

Twenty-four mutations were identified amongst the 41 samples: 14 from exon 5; 0 from exon 6; 4 from exon 7 and; 6 from exon 8. In an effort to avoid erroneous mutations being detected I set a threshold for counting a mutation as 'real' if it was detected in more than two repeats (barcodes) of the same sample AND in loci where the minimum depth of coverage was 20 reads. Thus in order to be counted the minimum requirement would be that the mutation occurred in 2/20 reads (10%) AND

in at least 2 of the repeats. In cases where the mutation was observed but did not meet the above criteria, they were recorded as 'possible' mutations.

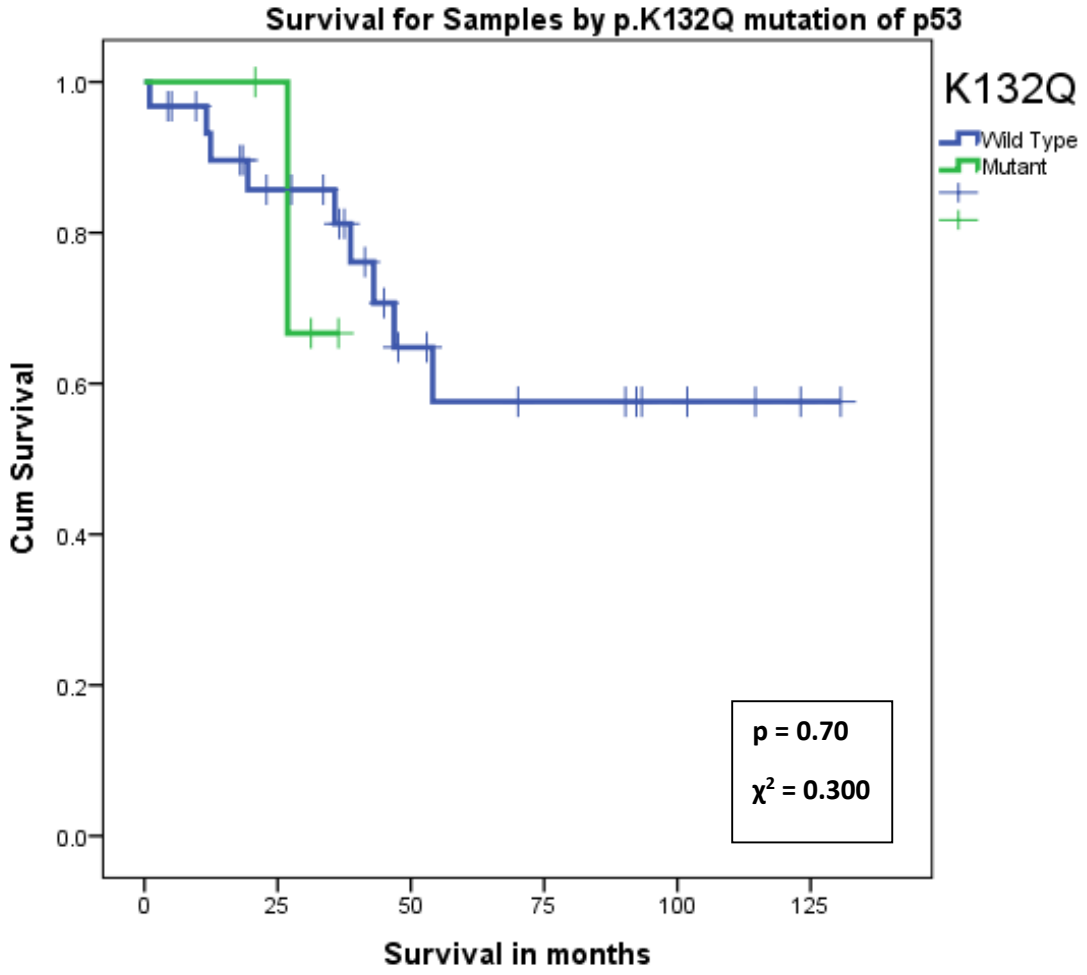
Exon 5 of *TP53*

Four of the 14 mutations identified occurred in at least three different samples: p.K132Q; p.M160L; p.K164R and; p.K164T. The relative frequencies of mutations found in exon 5 are expressed in Table . In two cases mutations were identified which did not fulfil the inclusion criteria outlined above and have been listed as 'possible' mutations.

Table 28: Frequency of mutations detected by exon.

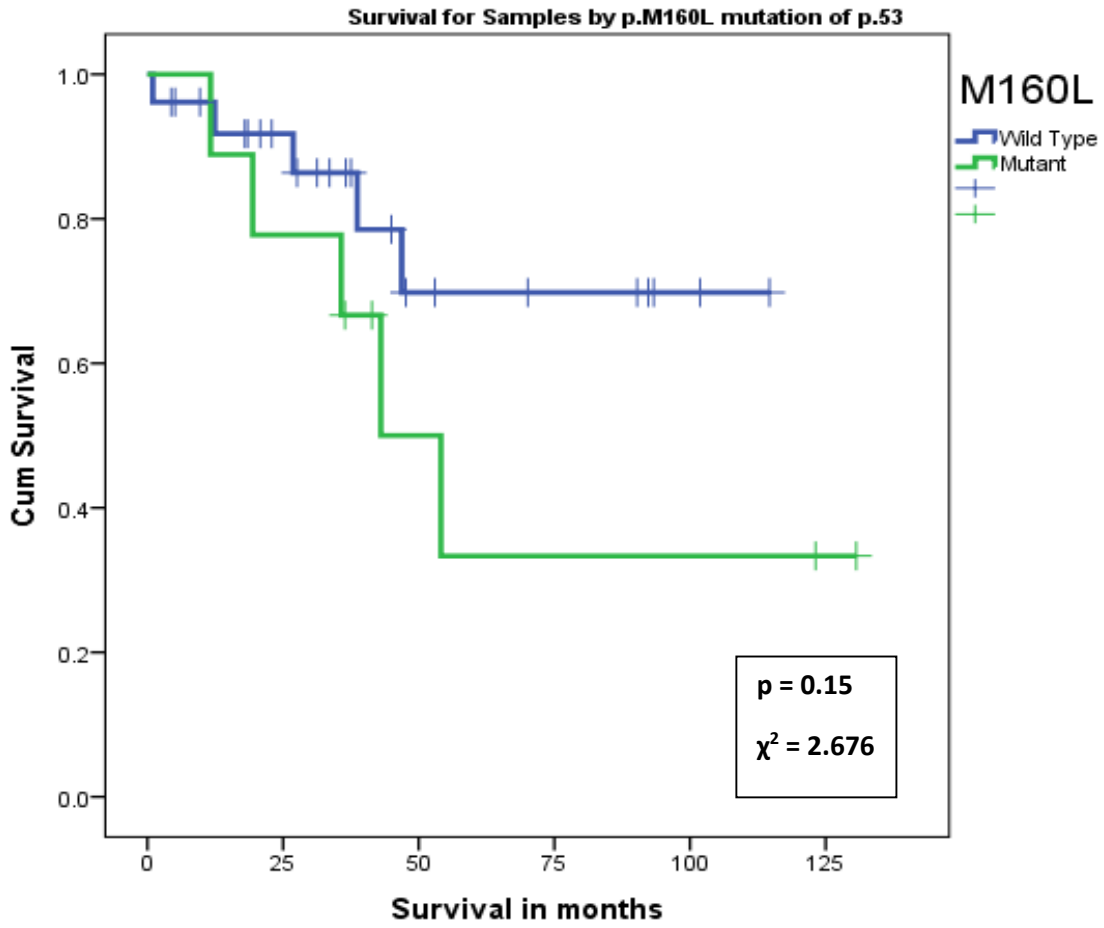
Exon	p. Notation	g. Notation	Occurrences (n=41)	Published Somatic Count
5	Y126D	7578554 A>C	1	6
	L130F	7578542 G>A	2	15
	K132Q	**7578536 T>G**	**4**	**19**
	P153A	7578473 G>C	1	3
	G154R	7578470 C>G	1	0
	T155P	7578467 T>G	1	23
	A159P	7578455 C>G	2	31
	M160L	**7578452 T>G**	**10**	5
	M160I	*7578450 C>T*	*0*	*3 (Possible Mutation)*
	K164R	**7578439 T>C**	**9**	2
	K164T	**7578439 T>G**	**3**	2
	M169I	*7578423 C>T*	*0*	*13 (Possible Mutation)*
	E171G	7578418 T>C	2	8
	C176S	7578404 A>T	1	20
7	V216A	7578202 A>G	1	0
	V217G	7578199 A>C	1	0
	S240N	7577562 C>T	2	4
	I251L	**7577530 T>G**	**10**	6
8	**L264R**	**7577147 A>C**	**7**	7
	E271G	**7577126 T>C**	**9**	5
	E271A	7577126 T>G	1	2
	E271V	7577126 T>A	1	0
	R273H	**7577120 C>T**	**4**	851
	P278S	7577106 G>A	1	91

Each of the mutations identified were analysed against overall survival using the Kaplan-Meier method with Log-rank used to measure significance. None of the mutations were found to significantly affect survival. The analyses for the four mutations present in >3 cases are reproduced in Figures 31 – 34.



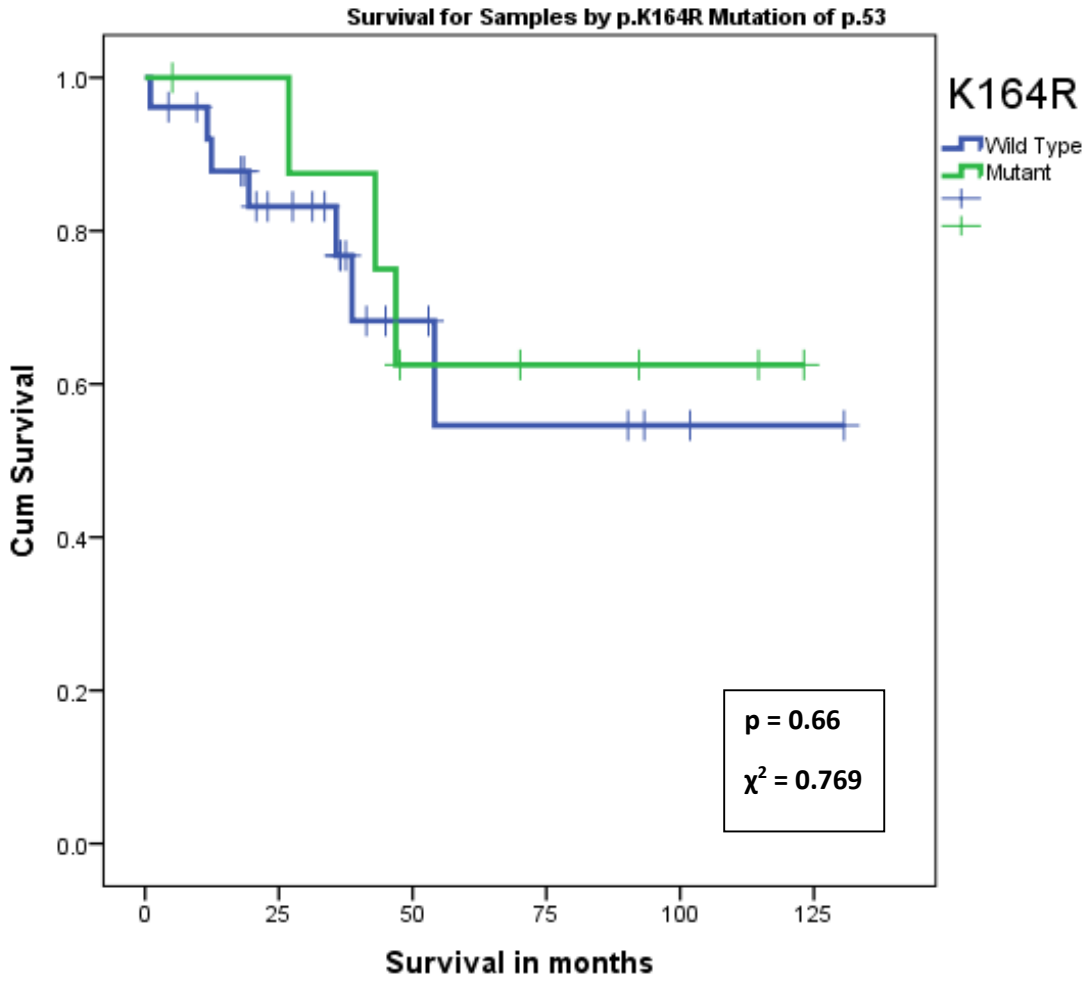
	0	25	50	75	100	125
Wild Type	36	25	12	8	4	1
Mutant	4	3	0	0	0	0

Figure 31: Kaplan-Meier survival analysis by mutant or wild type p.K132Q.



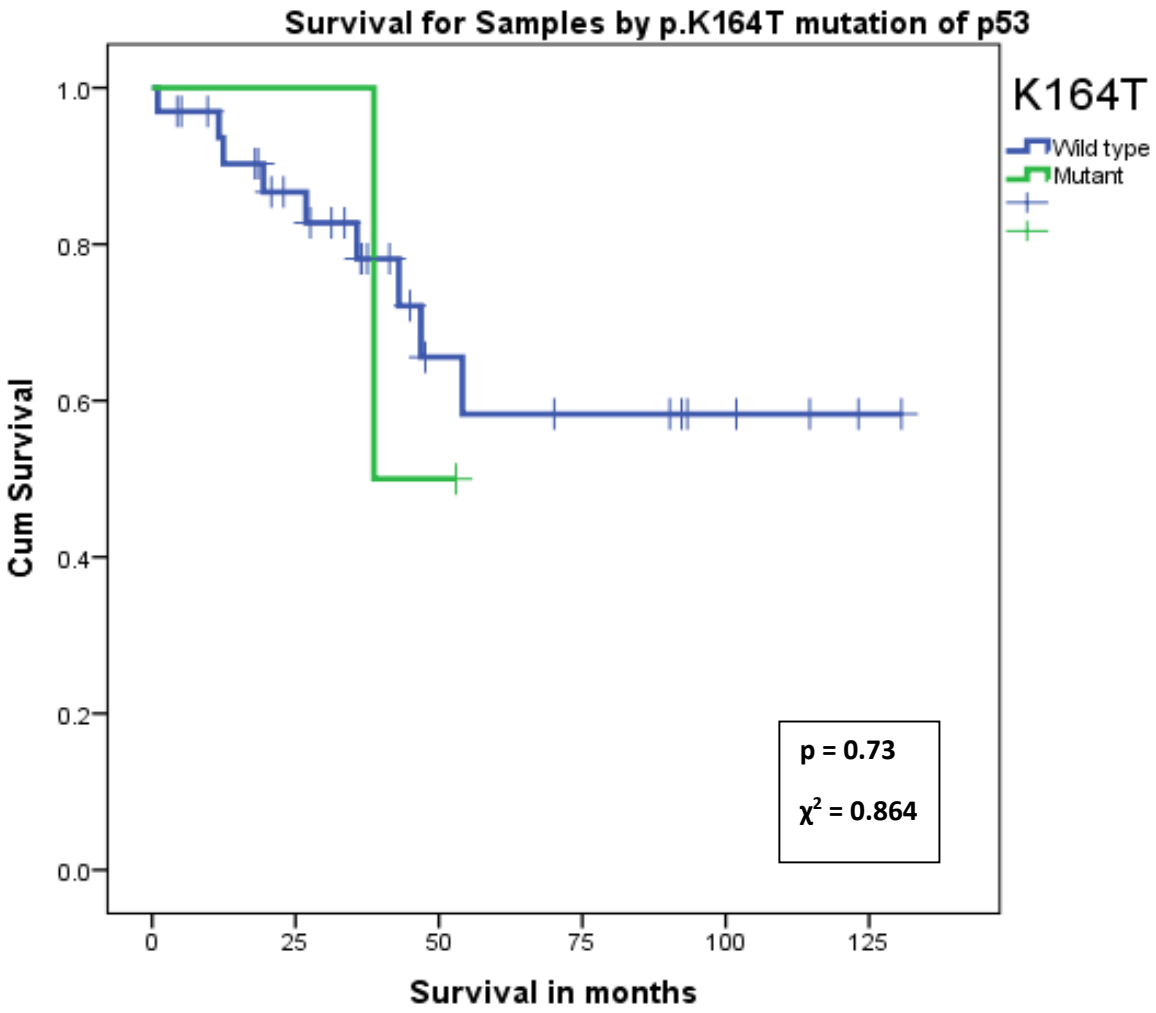
	0	25	50	75	100	125
Wild Type	30	20	9	6	2	0
Mutant	10	8	3	2	2	1

Figure 32: Kaplan-Meier survival analysis by mutant or wild type p.M160L.



	0	25	50	75	100	125
Wild Type	32	21	9	5	2	1
Mutant	8	7	3	3	2	0

Figure 33: Kaplan-Meier survival analysis by mutant or wild type p.K164R.



	0	25	50	75	100	125
Wild Type	37	25	11	8	4	1
Mutant	3	2	1	0	0	0

Figure 34: Kaplan-Meier survival analysis by mutant or wild type p.K164T.

In addition each of these mutations were assessed for any relationship to demographic factors (age, alcohol, smoking, diabetes) and pathological factors (tumour status, nodal status, presence of metastases, weight loss, epithelial sub-type) as well as the MUC staining described in 4.2.1 - Immunohistochemistry Methods. These analyses are presented in Tables 29 – 32.

Table 29: Results for p.K132Q.

p.K132Q				
Variable		Wild Type	Mutant	p.
Gender	Female	15	1	.488
	Male	22	3	
MUC1	Negative	13	3	.316
	Positive	7	0	
MUC2	Negative	11	1	.466
	Positive	9	2	
MUC5AC	Negative	1	0	.870
	Positive	19	3	
MUC6	Negative	10	2	.534
	Positive	10	1	
Epithelial subtype	Unknown	19	1	.514
	Gastric	6	1	
	Intestinal	5	2	
	Mixed	4	0	
	Oncocytic	1	0	
	Pancreatobiliary	2	0	
Tumour Status	0	10	0	.205
	1	1	1	
	2	1	0	
	3	12	2	
Nodal Status	0	19	1	.156
	1	5	2	
Mets	0	20	3	.770
	1	2	0	
Resection Margin	0	23	3	.531
	1	2	1	
	2	1	0	
Primary or Secondary Tumour	Unknown	1	0	.735
	Primary	32	4	
	Recurrence	4	0	
Location of Tumour	Unknown	1	0	.666
	Body	5	0	
	Head	18	2	
	Tail	8	2	
	Whole	5	0	
Diabetes	None	27	3	.612
	Diet	3	0	

	Insulin	3	1	
	Metformin	4	0	
Smoker	Former	5	2	.154
	No	25	2	
	Yes	7	0	
Alcohol	Unknown	1	0	.370
	Daily	6	2	
	Former	1	0	
	No	16	0	
	Occasionally	13	2	
Weight loss	No	18	4	.072
	Yes	19	0	
Peritoneal mets	No	36	4	.902
	Yes	1	0	
Grade of Dysplasia	Unknown	19	1	.666
	Cancer	3	1	
	HGD	2	0	
	IGD	4	1	
	LGD	9	1	

Table 30: Results for p.M160L.

p.M160L				
Variable		Wild Type	Mutant	p.
Gender	Female	12	4	.612
	Male	19	6	
MUC1	Negative	11	5	.130
	Positive	7	0	
MUC2	Negative	10	2	.455
	Positive	8	3	
MUC5AC	Negative	0	1	.217
	Positive	18	4	
MUC6	Negative	8	4	.185
	Positive	10	1	
Epithelial subtype	Unknown	15	5	.605
	Gastric	5	2	
	Intestinal	4	3	
	Mixed	4	0	
	Oncocytic	1	0	
	Pancreatobiliary	2	0	
Tumour Status	0	9	1	**.037**
	1	0	2	

	2	0	1	
	3	9	5	
Nodal Status	0	14	6	.429
	1	4	3	
Mets	0	16	7	.547
	1	1	1	
Resection Margin	0	19	7	.805
	1	2	1	
	2	1	0	
Primary or Secondary Tumour	Unknown	1	0	.399
	Primary	26	10	
	Recurrence	4	0	
Location of Tumour	Unknown	1	0	.597
	Body	5	0	
	Head	15	5	
	Tail	7	3	
	Whole	3	2	
Diabetes	None	20	10	.183
	Diet	3	0	
	Insulin	4	0	
	Metformin	4	0	
Smoker	Former	6	1	.555
	No	19	8	
	Yes	6	1	
Alcohol	Unknown	0	1	.331
	Daily	5	3	
	Former	1	0	
	No	13	3	
	Occasionally	12	3	
Weight loss	No	18	4	.264
	Yes	13	6	
Peritoneal mets	No	31	9	.244
	Yes	0	1	
Grade of Dysplasia	Unknown	15	5	
	Cancer	3	1	.842
	HGD	2	0	
	IGD	3	2	
	LGD	8	2	

Table 31: Results for p.K164R.

p.K164R				
Variable		Wild Type	Mutant	p.
Gender	Female	14	2	.220
	Male	18	7	
MUC1	Negative	14	2	.352
	Positive	5	2	
MUC2	Negative	11	1	.261
	Positive	8	3	
MUC5AC	Negative	1	0	.826
	Positive	18	4	
MUC6	Negative	9	3	.329
	Positive	10	1	
Epithelial subtype	Unknown	15	5	.627
	Gastric	7	0	
	Intestinal	5	2	
	Mixed	3	1	
	Oncocytic	1	0	
	Pancreatobiliary	1	1	
Tumour Status	0	7	3	.843
	1	1	1	
	2	1	0	
	3	10	4	
Nodal Status	0	15	5	.332
	1	4	3	
Mets	0	15	8	.453
	1	2	0	
Resection Margin	0	18	8	.432
	1	3	0	
	2	1	0	
Primary or Secondary Tumour	Unknown	1	0	.859
	Primary	28	8	
	Recurrence	3	1	
Location of Tumour	Unknown	1	0	.597
	Body	3	2	
	Head	15	5	
	Tail	8	2	
	Whole	5	0	
Diabetes	None	25	5	**.048**
	Diet	3	0	
	Insulin	1	3	
	Metformin	3	1	
Smoker	Former	5	2	.762

	No	22	5	
	Yes	5	2	
Alcohol	Unknown	1	0	.277
	Daily	8	0	
	Former	1	0	
	No	10	6	
	Occasionally	12	3	
Weight loss	No	20	2	**.038**
	Yes	12	7	
Peritoneal mets	No	31	9	.780
	Yes	1	0	
Grade of Dysplasia	Unknown	15	5	.501
	Cancer	2	2	
	HGD	2	0	
	IGD	4	1	
	LGD	9	1	

Table 32: Results for p.K164T.

p.K164T				
Variable		Wild Type	Mutant	p.
Gender	Female	16	0	.216
	Male	22	3	
MUC1	Negative	16	0	.304
	Positive	6	1	
MUC2	Negative	12	0	.478
	Positive	10	1	
MUC5AC	Negative	1	0	.957
	Positive	21	1	
MUC6	Negative	12	0	.478
	Positive	10	1	
Epithelial subtype	Unknown	18	2	.639
	Gastric	7	0	
	Intestinal	7	0	
	Mixed	3	1	
	Oncocytic	1	0	
	Pancreatobiliary	2	0	
Tumour Status	0	8	2	.706
	1	2	0	
	2	1	0	
	3	13	1	
Nodal Status	0	17	3	.390
	1	7	0	
Mets	0	20	3	.770

	1	2	0	
Resection Margin	0	23	3	.774
	1	3	0	
	2	1	0	
Primary or Secondary Tumour	Unknown	1	0	.799
	Primary	33	3	
	Recurrence	4	0	
Location of Tumour	Unknown	1	0	.750
	Body	4	1	
	Head	19	1	
	Tail	9	1	
	Whole	5	0	
Diabetes	None	28	2	.491
	Diet	3	0	
	Insulin	4	0	
	Metformin	3	1	
Smoker	Former	7	0	.590
	No	25	2	
	Yes	6	1	
Alcohol	Unknown	1	0	.806
	Daily	8	0	
	Former	1	0	
	No	15	1	
	Occasionally	13	2	
Weight loss	No	20	2	.556
	Yes	18	1	
Peritoneal mets	No	37	3	.927
	Yes	1	0	
Grade of Dysplasia	Unknown	18	2	.131
	Cancer	4	0	
	HGD	1	1	
	IGD	5	0	
	LGD	10	0	

The only findings of significance were an association between increased tumour status and the presence of p.M160L (p=.037) and weight loss was more likely in patients who expressed p.K164R mutation (p=.038). There was also a predisposition to diabetes amongst those who had mutant p.K164R (p=0.48). However, in all of these cases correction for multiple testing would make these differences insignificant.

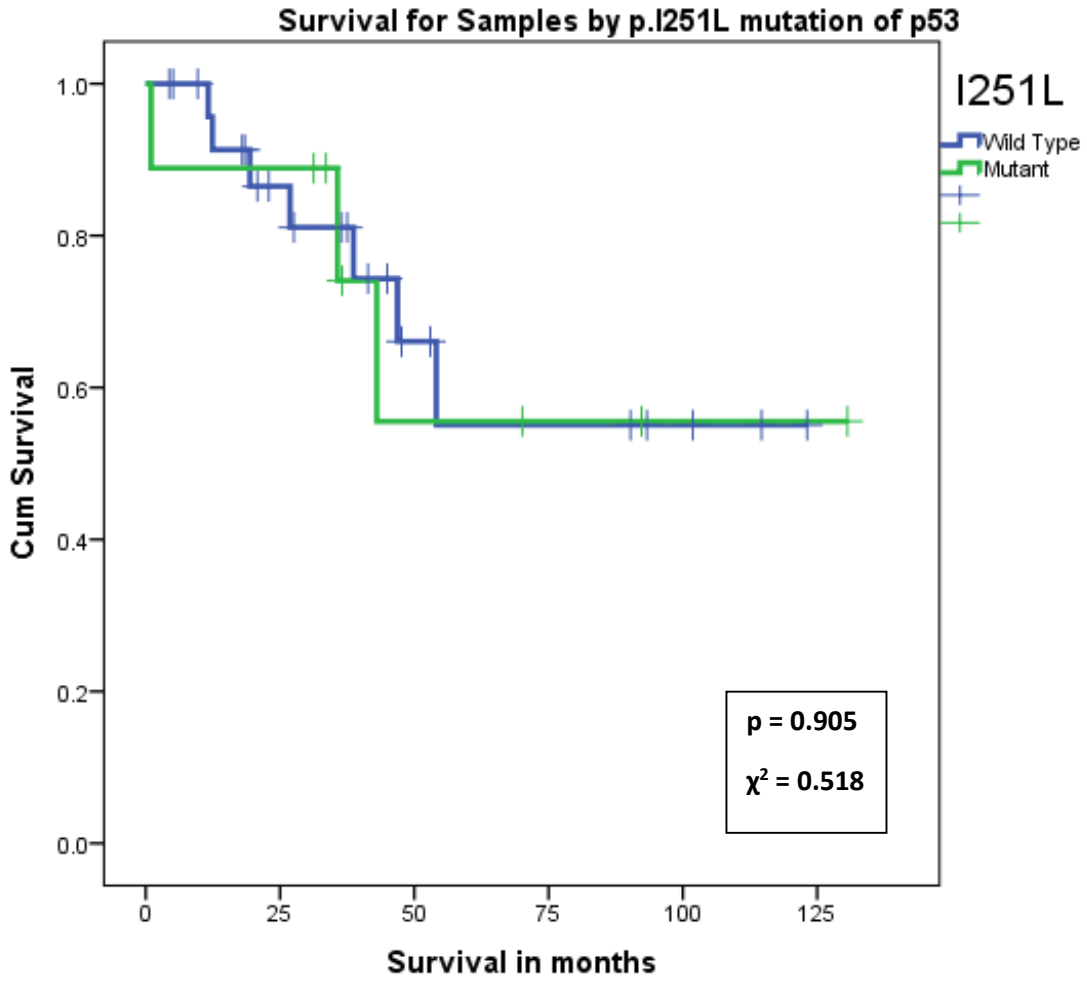
Exon 6 of *TP53*

No mutations were demonstrated to occur in exon 6.

Exon 7 of *TP53*

One of the 4 mutations identified occurred in at least three different samples: p.I251L. The relative frequencies of mutations found in exon 7 are expressed in Table 28.

Each of the mutations identified were analysed against overall survival using the Kaplan-Meier method with Log-rank used to measure significance. The presence pf p.I251L was not found to significantly affect survival.



	0	25	50	75	100	125
Wild Type	31	20	10	6	3	0
Mutant	9	8	4	3	2	1

Figure 35: Kaplan-Meier survival analysis by mutant or wild type p.I251L.

In addition p.I251L was assessed for any relationship to demographic factors (age, alcohol, smoking, and diabetes) and pathological factors (tumour status, nodal status, presence of metastases, weight loss, and epithelial sub-type) as well as the MUC staining described in 4.2.1 - Immunohistochemistry Methods. These analyses are presented in Table 33.

Table 33: Results for p.I251L.

p.I251L				
Variable		Wild Type	Mutant	p.
Gender	Female	14	2	.148
	Male	17	8	
MUC1	Negative	14	2	.352
	Positive	5	2	
MUC2	Negative	11	1	.261
	Positive	8	3	
MUC5AC	Negative	1	0	.826
	Positive	18	4	
MUC6	Negative	9	3	.329
	Positive	10	1	
Epithelial subtype	Unknown	14	6	.622
	Gastric	6	1	
	Intestinal	6	1	
	Mixed	2	2	
	Oncocytic	1	0	
	Pancreatobiliary	2	0	
Tumour Status	0	6	4	.242
	1	2	0	
	2	0	1	
	3	11	3	
Nodal Status	0	14	6	.668
	1	5	2	
Mets	0	16	7	.510
	1	2	0	
Resection Margin	0	20	6	.789
	1	2	1	
	2	1	0	
Primary or Secondary Tumour	Unknown	1	0	.848
	Primary	27	9	
	Recurrence	3	1	
Location of Tumour	Unknown	1	0	.928
	Body	4	1	
	Head	14	6	

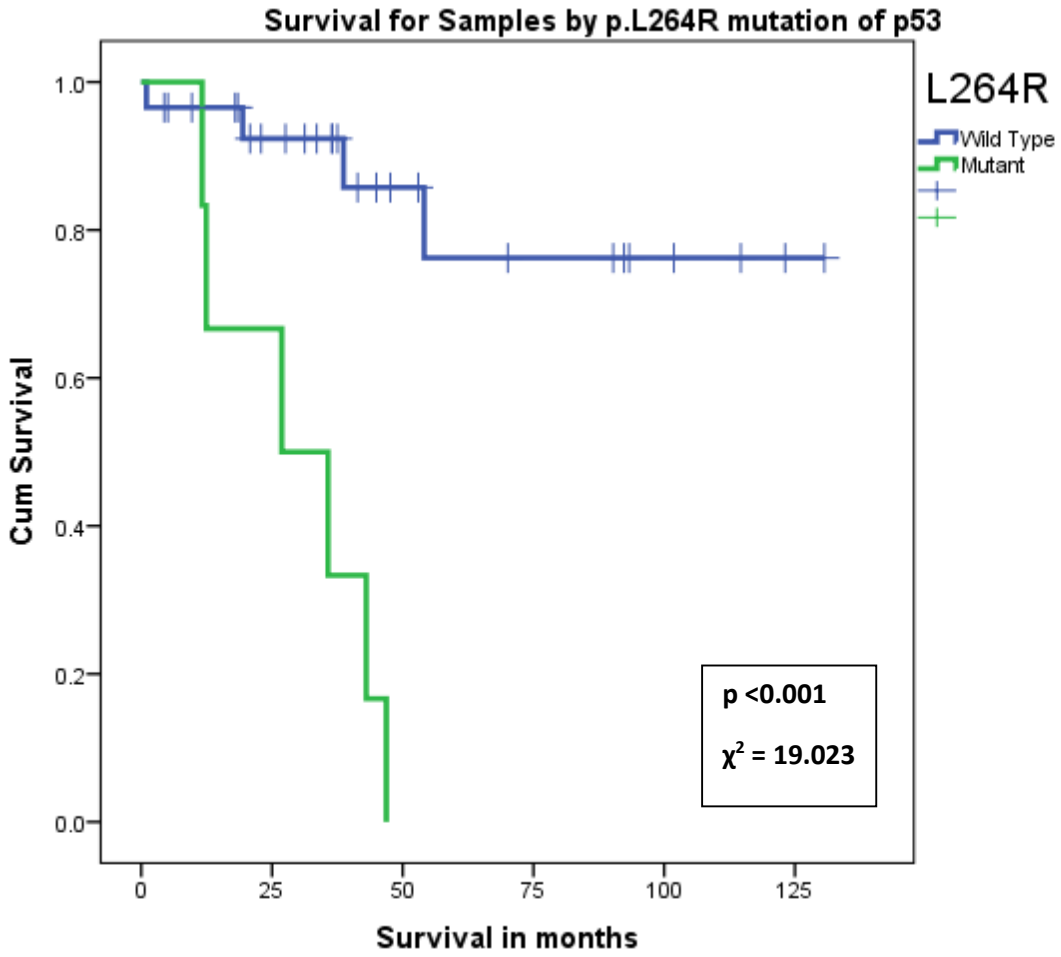
	Tail	8	2	
	Whole	4	1	
Diabetes	None	22	8	.288
	Diet	3	0	
	Insulin	2	2	
	Metformin	4	0	
Smoker	Former	6	1	**.006**
	No	23	4	
	Yes	2	5	
Alcohol	Unknown	1	0	.789
	Daily	5	3	
	Former	1	0	
	No	13	3	
	Occasionally	11	4	
Weight loss	No	17	5	.537
	Yes	14	5	
Peritoneal mets	No	30	10	.756
	Yes	1	0	
Grade of Dysplasia	Unknown	14	6	.145
	Cancer	4	0	
	HGD	2	0	
	IGD	2	3	
	LGD	9	1	

The only finding of significance was an increased incidence of p.I251L being found in patients who declared themselves as current smokers (p=0.006).

Exon 8 of *TP53*

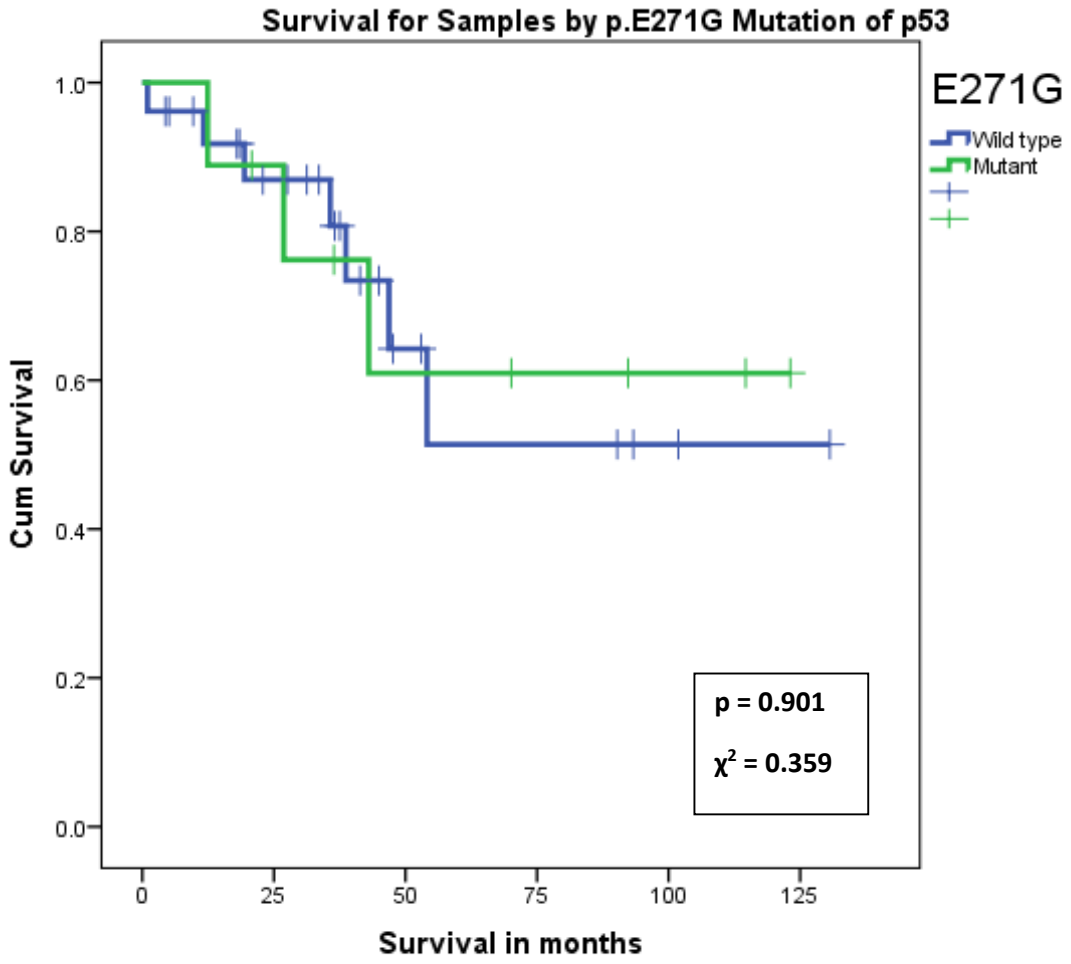
Three of the 6 mutations identified occurred in at least three different samples: p.L264R; p.E271G and; p.R273H. The relative frequencies of mutations found in exon 8 are expressed in Table .

Each of the mutations identified were analysed against overall survival using the Kaplan-Meier method with Log-rank used to measure significance. **A single mutation, p.L264R, was found to significantly predict poor survival, p<0.001**.



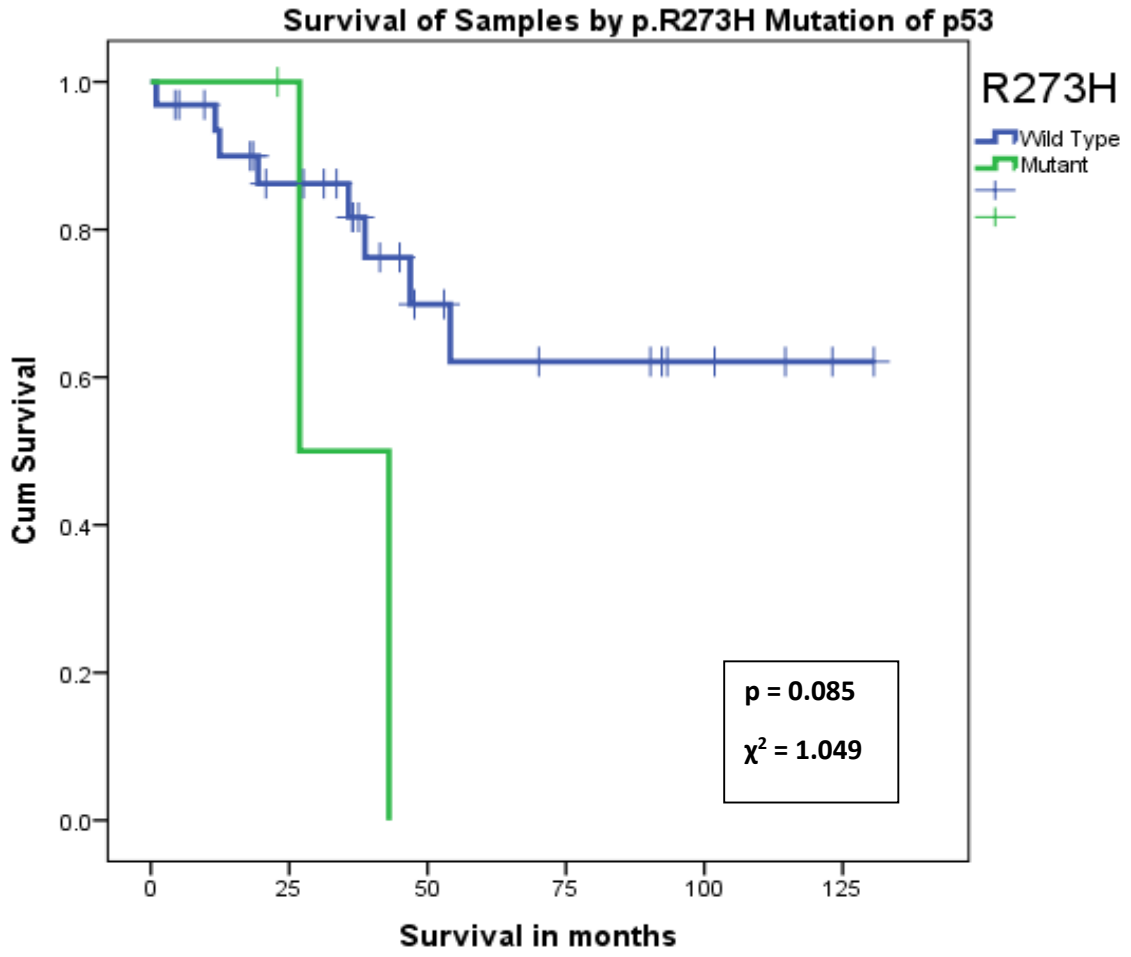
	0	25	50	75	100	125
Wild Type	28	18	8	6	3	1
Mutant	6	3	0	0	0	0

Figure 36: Kaplan-Meier survival analysis by mutant or wild type p.L264R.



	0	25	50	75	100	125
Wild Type	32	21	9	5	2	0
Mutant	8	6	3	3	2	0

Figure 37: Kaplan-Meier survival analysis by mutant or wild type p.E271G.



	0	25	50	75	100	125
Wild Type	36	25	11	7	4	1
Mutant	4	3	0	0	0	0

Figure 38: Kaplan-Meier survival analysis by mutant or wild type p.R273H.

In addition each of these mutations were assessed for any relationship to demographic factors (age, alcohol, smoking, diabetes) and pathological factors (tumour status, nodal status, presence of metastases, weight loss, and epithelial sub-type) as well as the MUC staining described in 4.2.1 - Immunohistochemistry Methods. These analyses are presented Tables 34 - 36.

Table 34: Results for p.L234R

p.L264R				
Variable		Wild Type	Mutant	p.
Gender	Female	14	2	.431
	Male	20	5	
MUC1	Negative	13	3	.316

	Positive	7	0	
MUC2	Negative	11	1	.466
	Positive	9	2	
MUC5	Negative	1	0	.870
	Positive	19	3	
MUC6	Negative	10	2	.534
	Positive	10	1	
Epithelial subtype	Unknown	15	5	.490
	Gastric	7	0	
	Intestinal	5	2	
	Mixed	4	0	
	Oncocytic	1	0	
	Pancreatobiliary	2	0	
Tumour Status	0	9	1	.216
	1	2	0	
	2	1	0	
	3	8	6	
Nodal Status	0	18	2	**.005**
	1	2	5	
Mets	0	19	4	**.050**
	1	0	2	
Resection Margin	0	19	7	.495
	1	3	0	
	2	1	0	
Primary or Secondary Tumour	Unknown	1	0	.556
	Primary	29	7	
	Recurrence	4	0	
Location of Tumour	Unknown	1	0	.836
	Body	4	1	
	Head	16	4	
	Tail	8	2	
	Whole	5	0	
Diabetes	None	25	5	.807
	Diet	3	0	
	Insulin	3	1	
	Metformin	3	1	
Smoker	Former	6	1	.943
	No	22	5	
	Yes	6	1	
Alcohol	Unknown	1	0	.846
	Daily	7	1	
	Former	1	0	
	No	12	4	
	Occasionally	13	2	

Weight loss	No	17	5	.271
	Yes	17	2	
Peritoneal mets	No	34	6	.171
	Yes	0	1	
Grade of Dysplasia	Unknown	15	5	.468
	Cancer	3	1	
	HGD	2	0	
	IGD	4	1	
	LGD	10	0	

Table 35: Results for p.E271G.

p.E271G				
Variable		Wild Type	Mutant	p.
Gender	Female	14	2	.220
	Male	18	7	
MUC1	Negative	12	4	.508
	Positive	6	1	
MUC2	Negative	11	1	.131
	Positive	7	4	
MUC5	Negative	1	0	.783
	Positive	17	5	
MUC6	Negative	9	3	.545
	Positive	9	2	
Epithelial subtype	Unknown	16	4	.710
	Gastric	6	1	
	Intestinal	4	3	
	Mixed	3	1	
	Oncocytic	1	0	
	Pancreatobiliary	2	0	
Tumour Status	0	8	2	.094
	1	0	2	
	2	1	0	
	3	11	3	
Nodal Status	0	16	4	.241
	1	4	3	
Mets	0	17	6	.490
	1	1	1	
Resection Margin	0	18	8	.432
	1	3	0	
	2	1	0	
Primary or Secondary Tumour	Unknown	1	0	.859
	Primary	28	8	
	Recurrence	3	1	

Location of Tumour	Unknown	1	0	.376
	Body	5	0	
	Head	14	6	
	Tail	7	3	
	Whole	5	0	
Diabetes	None	25	5	**.048**
	Diet	3	0	
	Insulin	1	3	
	Metformin	3	1	
Smoker	Former	4	3	.251
	No	23	4	
	Yes	5	2	
Alcohol	Unknown	1	0	.925
	Daily	6	2	
	Former	1	0	
	No	13	3	
	Occasionally	11	4	
Weight loss	No	18	4	.400
	Yes	14	5	
Peritoneal mets	No	31	9	.780
	Yes	1	0	
Grade of Dysplasia	Unknown	16	4	.754
	Cancer	3	1	
	HGD	2	0	
	IGD	3	2	
	LGD	8	2	

Table 36: Results for p.R273H.

p.R273H				
Variable		Wild Type	Mutant	p.
Gender	Female	14	2	.512
	Male	23	2	
MUC1	Negative	14	2	.684
	Positive	6	1	
MUC2	Negative	11	1	.466
	Positive	9	2	
MUC5	Negative	1	0	.870
	Positive	19	3	
MUC6	Negative	10	2	.534
	Positive	10	1	
Epithelial subtype	Unknown	19	1	.818
	Gastric	6	1	
	Intestinal	6	1	

	Mixed	3	1	
	Oncocytic	1	0	
	Pancreatobiliary	2	0	
Tumour Status	0	10	0	.571
	1	2	0	
	2	1	0	
	3	12	2	
Nodal Status	0	20	0	.060
	1	5	2	
Mets	0	21	2	.843
	1	2	0	
Resection Margin	0	24	2	.848
	1	3	0	
	2	1	0	
Primary or Secondary Tumour	Unknown	1	0	.735
	Primary	32	4	
	Recurrence	4	0	
Location of Tumour	Unknown	1	0	.870
	Body	5	0	
	Head	18	2	
	Tail	9	1	
	Whole	4	1	
Diabetes	None	27	3	.612
	Diet	3	0	
	Insulin	3	1	
	Metformin	4	0	
Smoker	Former	6	1	.614
	No	24	3	
	Yes	7	0	
Alcohol	Unknown	1	0	**.031**
	Daily	8	0	
	Former	0	1	
	No	15	1	
	Occasionally	13	2	
Weight loss	No	20	2	.639
	Yes	17	2	
Peritoneal mets	No	36	4	.902
	Yes	1	0	
Grade of Dysplasia	Unknown	19	1	.475
	Cancer	3	1	
	HGD	2	0	
	IGD	5	0	

	LGD	8	2	

In addition to the association between poor survival and p.L264R there was also an increased likelihood of patients with p.L264R having lymph node involvement (p=0.005) and metastases (p=0.050). This may well explain the poor overall survival. An increased incidence of diabetes was demonstrated amongst patients with p.E271G, p=0.048, after Bonferroni correction.

Samples with 2 or more concurrent mutations

Overall, of the 41 samples for whom Ion Torrent data is available, 18 had two or more concurrent mutations (Figure 39). When those patients with ≥2 mutations are compared to those with <2 there is no discernible difference in survival using the Kaplan-Meier method, p=0.921 (Figure 40). Similarly, even when the similar analysis is performed using only those patients who have 2 or more concurrent mutations consisting solely among the 8 commonest mutations there is no difference (p=0.348).

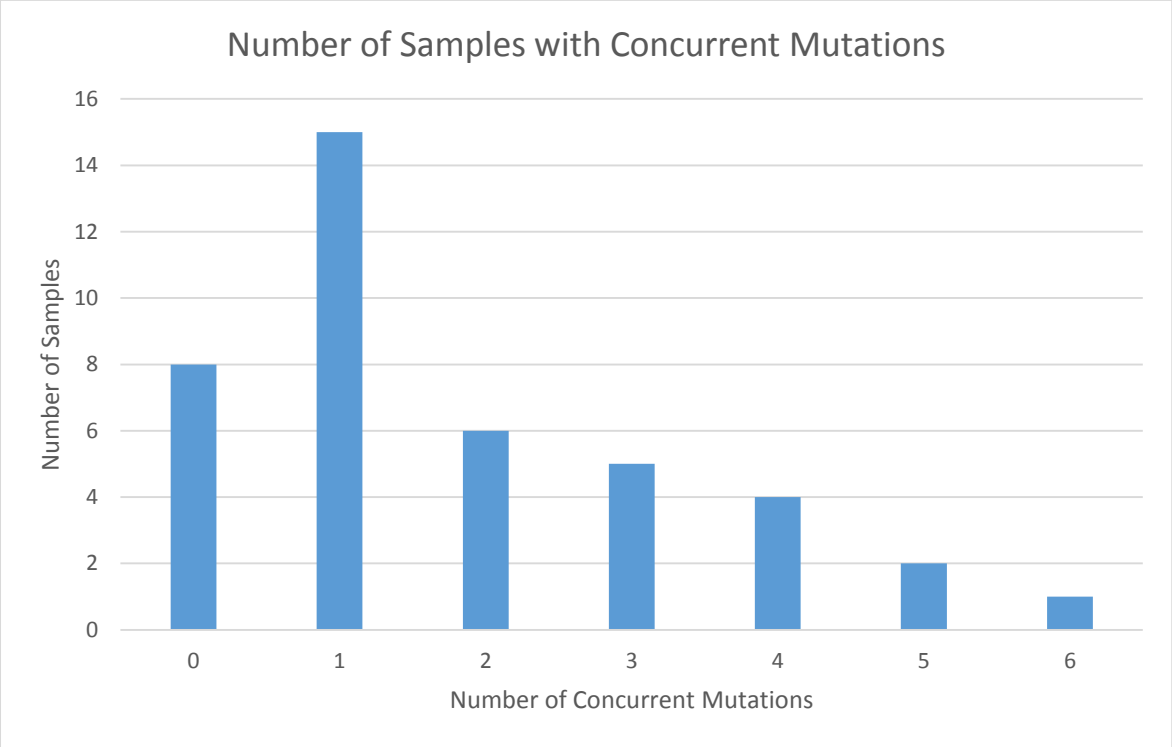
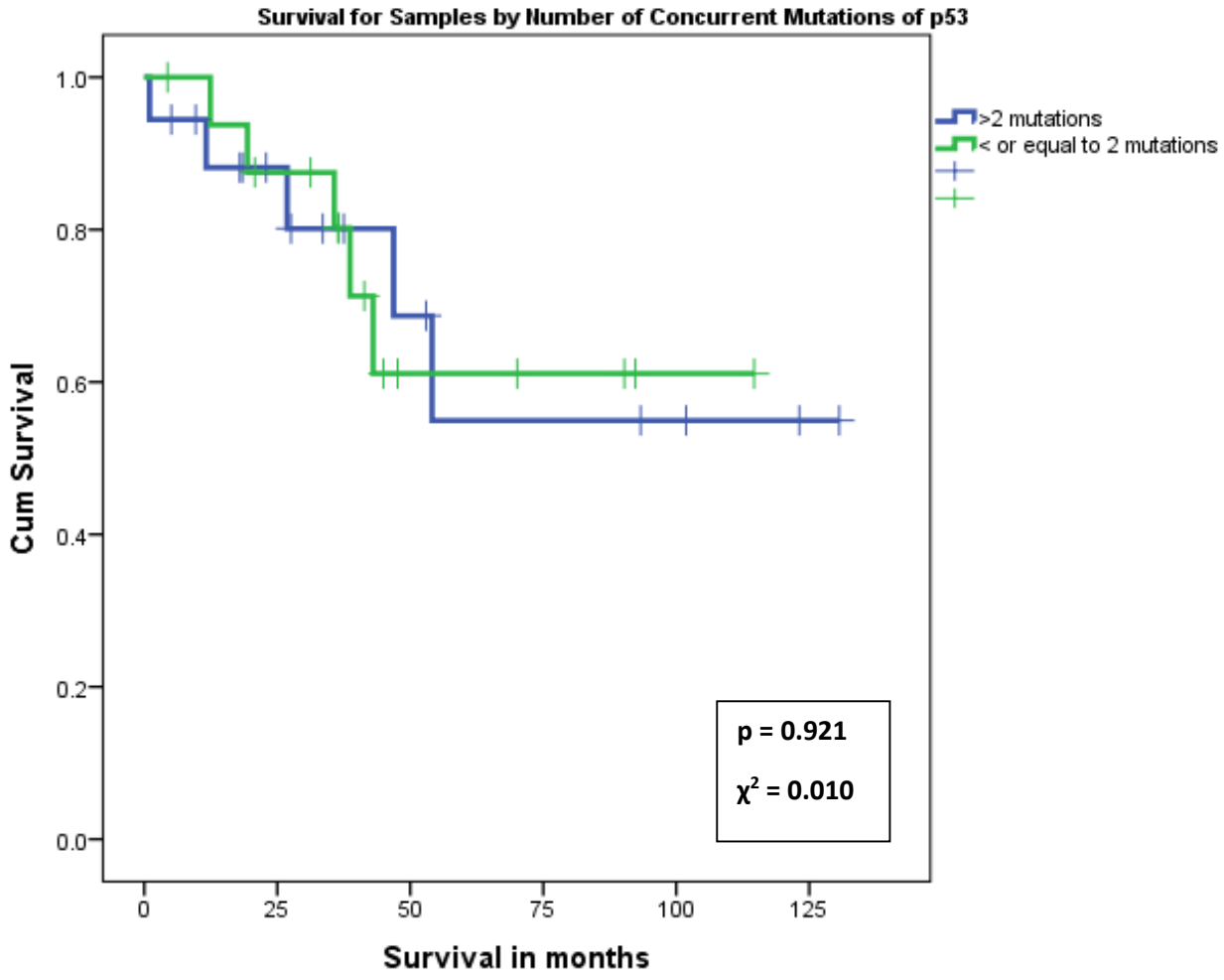
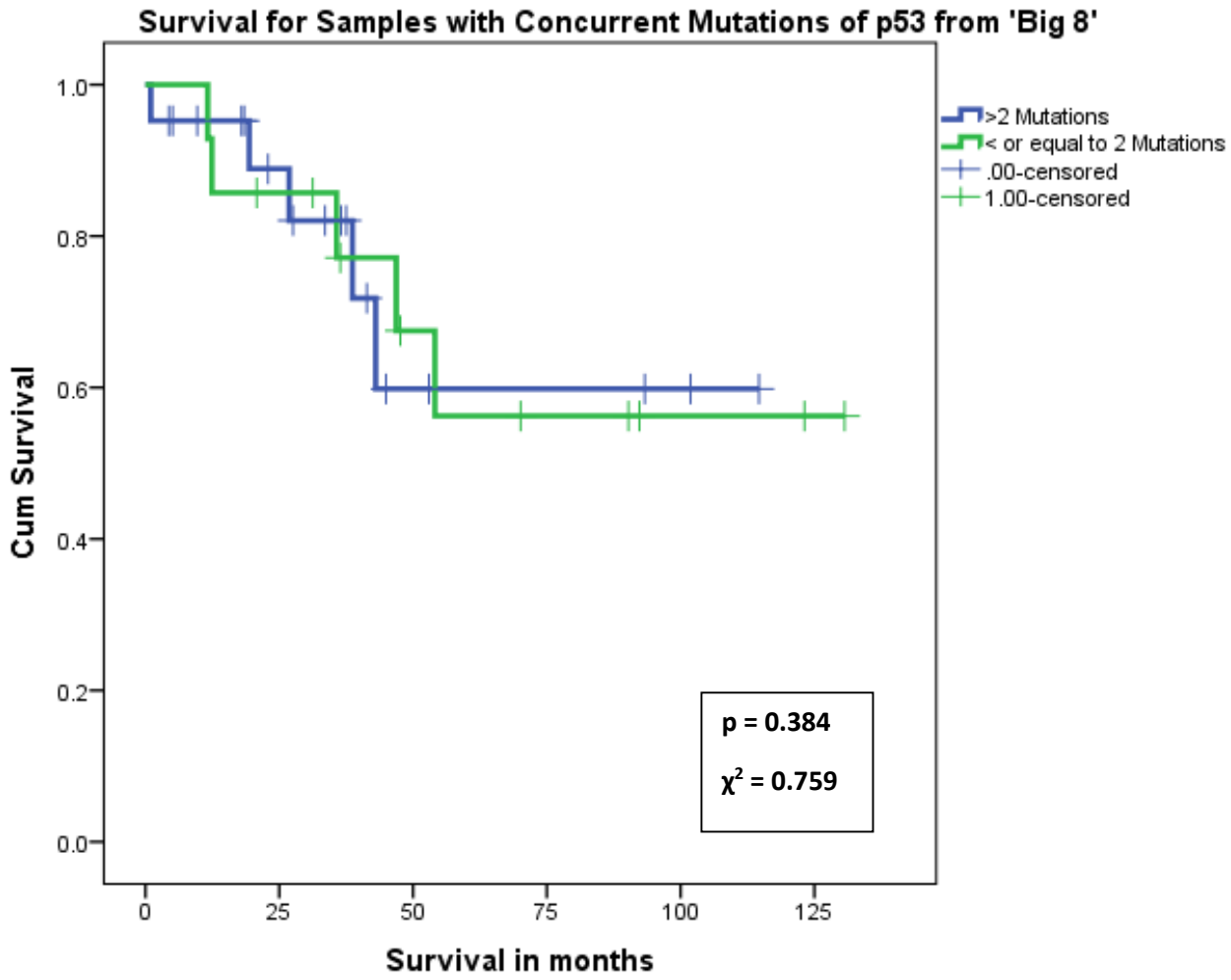


Figure 39: Graphical representation of number of samples with concurrent mutations.



	0	25	50	75	100	125
≥ 2	18	11	6	4	3	1
< 2	17	13	4	3	1	0

Figure 40: Kaplan-Meier analysis of survival when comparing those samples with < or equal to 2 concurrent mutations and >2.



	0	25	50	75	100	125
>2	14	11	5	4	3	1
≤ 2	21	13	5	3	1	0

Figure 41: Kaplan-Meier analysis of survival when comparing concurrent mutations from among the 8 identified in greater than 3 samples using Ion Torrent.

5.8 - Analysis of Individuals who Died

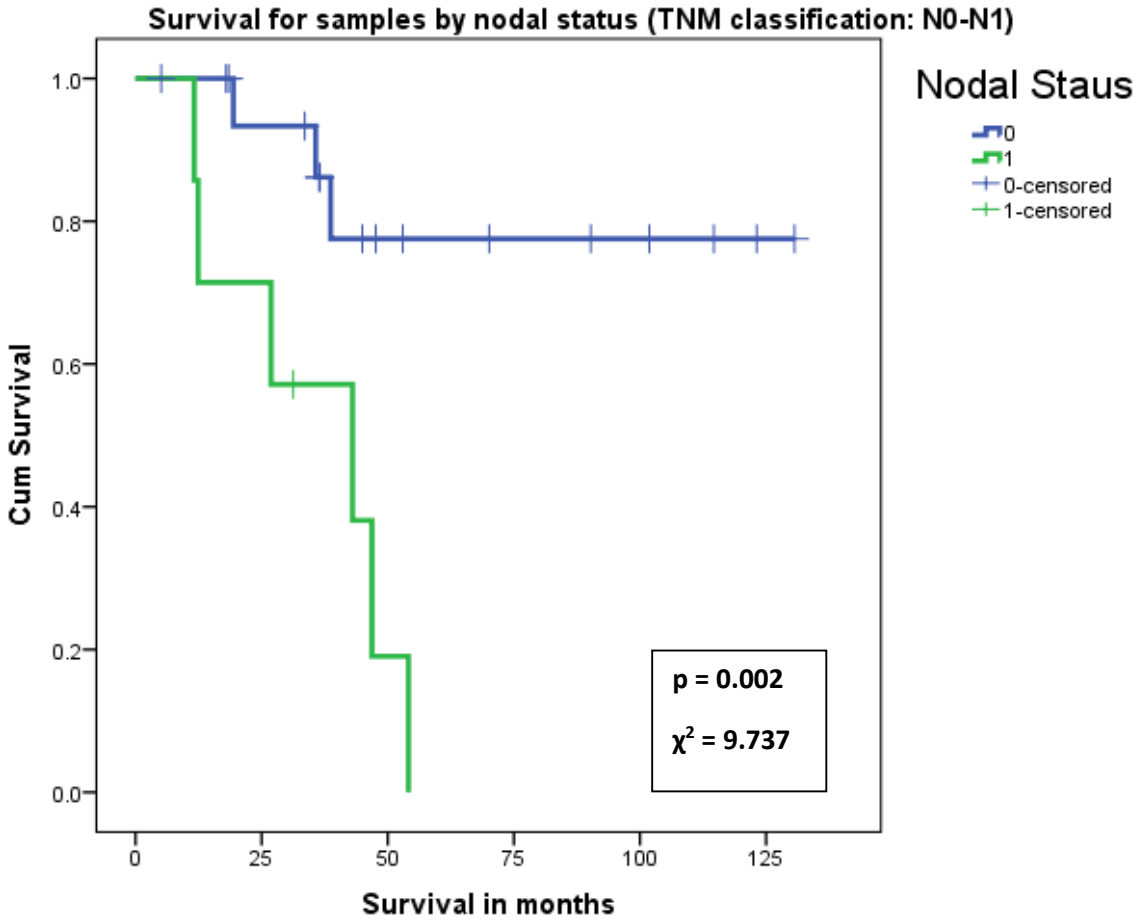
During the five years of follow-up 10 of the 41 patients (23%) in the Ion Torrent cohort died. One of these patients (CYST035) was recorded as a post-operative death following complications. The median age of these individuals was 68.40 years (IQR: 59.08 – 73.08), a comparative analysis of the demographics and pathological data for those patients who survived versus those patients who did not can be found in Table 37.

Table 37: Results by samples who have survived vs. those who have not.

Variable		Alive or Dead		p.
		alive	dead	
Male or Female	Female	13	3	0.39
	Male	18	7	
MUC1	Negative	12	4	0.51
	Positive	6	1	
MUC2	Negative	12	0	**0.01**
	Positive	6	5	
MUC5	Negative	0	1	0.22
	Positive	18	4	
MUC6	Negative	8	4	0.19
	Positive	10	1	
Epithelial subtype	Unknown	15	5	0.20
	Gastric	7	0	
	Intestinal	3	4	
	Mixed	3	1	
	Oncocytic	1	0	
	Pancreatobiliary	2	0	
Tumour Status	0	10	0	**0.01**
	1	2	0	
	2	1	0	
	3	5	9	
Nodal Status	0	17	3	**<0.005**
	1	1	6	
Mets	0	16	7	0.12
	1	0	2	
Resection Margin	0	18	8	0.80
	1	2	1	
	2	1	0	

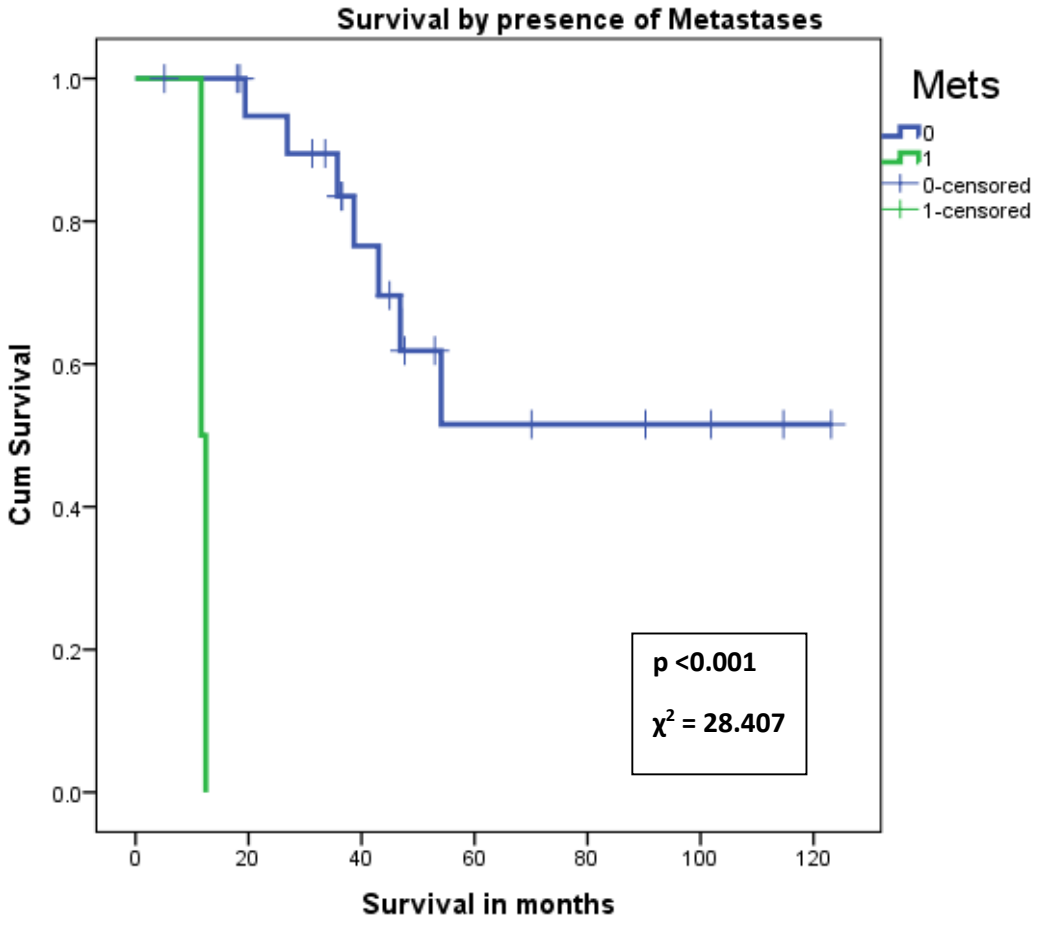
Primary or Secondary Tumour	Unknown	1	0	0.40
	Primary	26	10	
	Recurrence	4	0	
Location of Tumour	Unknown	1	0	0.89
	Body	3	2	
	Head	15	5	
	Tail	8	2	
	Whole	4	1	
Diabetes	None	22	8	0.79
	Diet	3	0	
	Insulin	3	1	
	Metformin	3	1	
Smoker	Former	6	1	0.56
	No	19	8	
	Yes	6	1	
Alcohol	Unknown	0	1	0.40
	Daily	7	1	
	Former	1	0	
	No	12	4	
	Occasion	11	4	
Weight loss	No	16	6	0.46
	Yes	15	4	
Peritoneal mets	No	31	9	0.24
	Yes	0	1	
median age (IQR)		64.8 (60.70 - 69.70)	68.4 (59.08 - 73.08)	0.736

Corresponding Kaplan-Meier analyses found that the only factors which affected survival were: increased nodal status ($p=0.002$) and the presence of metastases ($p<0.005$). These curves are demonstrated in Figures 42 and 43. Table 38 displays the demographic and pathological data for these 10 patients as well as their mutational status for each of the 8 most common mutations found among the Ion Torrent cohort.



	0	25	50	75	100	125
N0	18	14	7	5	4	1
N1	7	5	1	0	0	0

Figure 42: Kaplan-Meier analysis of survival when comparing different nodal status (TNM classification: N0-N1) for samples from the Ion Torrent cohort.



	0	25	50	75	100	125
M0	22	18	7	4	3	0
M1	2	0	0	0	0	0

Figure 43: Kaplan-Meier analysis of survival when comparing different metastasis status (TNM classification: M0-M1) for samples from the Ion Torrent cohort.

Of the 9 patients who died of pancreatic cancer, 6 had p.L264R mutation. Only one other patient was found to have this mutation (CYST063) who was lost to follow-up and so survival data cannot be obtained. As a consequence of these data, and having proven using Kaplan-Meier's method that p.L264R is significantly associated with survival we can calculate the sensitivity and specificity to this mutation to predict death by 5 years as follows:

		Survival to 5 years		
		Yes	No	TOTALS
p.L264R	Mutant	1	6	7
	Wild Type	30	4	34
	TOTALS	31	10	41

Sensitivity = 0.6 (95%CI: 0.27 – 0.86); Specificity = 0.97 (95%CI: 0.81 – 1); Positive Predictive Value = 0.86 (95%CI: 0.42 – 0.99).

Of the four patients who were wild-type for p.L264R but died, one (CYST035) died as a consequence of post-operative complications. The remaining three patients (CYST009, CYST010, CYST025) died of pancreatic cancer. It is worth noting, however, that one of these patients (CYST009) was 'borderline' for expression of p.L264R. The expressed percentage of reads which were mutant was 7.83%, below the 9% threshold defined in my methods.

If we remove CYST035 as a post-operative death and also exclude CYST063 as lost to follow-up (the only patient among the Ion Torrent cohort to be so) we find:

		Survival to 5 years		
		Yes	No	TOTALS
p.L264R	Mutant	0	6	6
	Wild Type	30	3	33
	TOTALS	30	9	39

Sensitivity = 0.67 (95%CI: 0.30 – 0.91); Specificity = 1 (95%CI: 0.86 – 1); Positive Predictive Value = 1 (95%CI: 0.52 – 1).

CYST -	7	9	10	14	15	18	21	22	25	35
Age (Yrs)	75	72	46	65	32	66	64	71	72	78
Gender	Male	Male	Male	Male	Male	Male	Female	Female	Female	Male
Survival (months)	12	39	54	27	43	47	12	36	19	1 (post-op)
HD histology	IPMC	IPMC	IPMC	IPMC	IPMC	IPMC	IPMC	IPMC	PDAC	IPMN
LV Histology	PDAC	IPMN	IPMN	IPMN	CP	CP	IPMN	CP	PDAC	IPMN
MUC1	-	**Positive**	Negative	Negative	-	-	Negative	-	-	Negative
MUC2	-	**Positive**	**Positive**	**Positive**	-	-	**Positive**	-	-	**Positive**
MUC5AC	-	**Positive**	***Patchy Pos***	**Positive**	-	-	**Positive**	-	-	**Positive**
MUC6	-	**Positive**	Negative	Negative	-	-	Negative	-	-	Negative
Epithelial subtype	-	Mixed	Mixed	Intestinal	-	-	Intestinal	-	-	Intestinal
Tumour Status	3	3	3	3	3	3	3	3	3	
Nodal Status	1	0	1	1	1	1	1	0	0	
Mets	1	0	0	0	0	0	1	0	0	
Resection Margin	0	0	0	0	0	0	0	0	1	
Primary or Secondary	Primary	Primary	Primary	Primary	Primary	Primary	Primary	Primary	Primary	Primary
Location	Head	Body	Whole	Tail	Head	Head	Tail	Head	Head	Body
Diabetes	Metformin	None	None	Insulin	None	None	None	None	None	None
Smoker	No	No	No	Former	No	No	No	No	No	Yes
Alcohol	Occasion	Occasion	Occasion	Occasion	No	No	No	No	Unknown	Daily
Weight Loss	No	No	Yes	No	Yes	No	No	Yes	Yes	No
Peritoneal mets	No	No	No	No	No	No	Yes	No	No	No
Dysplasia	-	HGD	Cancer	Cancer	-	-	IGD	-	-	LGD
K132Q	W/T	W/T	W/T	**Mutant**	W/T	W/T	W/T	W/T	W/T	W/T
M160L	W/T	W/T	**Mutant**	W/T	**Mutant**	W/T	**Mutant**	**Mutant**	**Mutant**	W/T
K164R	W/T	W/T	W/T	**Mutant**	**Mutant**	**Mutant**	W/T	W/T	W/T	W/T
K164T	W/T	**Mutant**	W/T	W/T	W/T	W/T	W/T	W/T	W/T	W/T
I251L	W/T	W/T	W/T	W/T	**Mutant**	W/T	W/T	**Mutant**	W/T	**Mutant**
E271G	**Mutant**	W/T	W/T	**Mutant**	**Mutant**	W/T	W/T	W/T	W/T	W/T
R273H	W/T	W/T	W/T	**Mutant**	**Mutant**	W/T	W/T	W/T	W/T	W/T
L264R	**Mutant**	W/T	W/T	**Mutant**	**Mutant**	**Mutant**	**Mutant**	**Mutant**	W/T	W/T

Table 38: Individual data for those samples coming from patients who have died.

5.9 - Validation

Once I had identified the significance on the p.L264R mutation I needed to try and develop a cheaper and easier method to detect it to enhance the clinical usefulness of p.L264R as a prognostic marker.

Given that I now had a single mutation I set about trying to create primers for use in a simple nested real time PCR which would be specific for this mutation. I could then use those samples which have been found to have the mutation on Ion Torrent analysis as the positive control when validating the PCR.

I used a simple primer design tool to design six sets of primers (three wild type and three mutant) which would create firstly a long fragment (739 bp) as round 1 and then using the amplified product of round one either a short product in round 2 (426 bp) or a long product in round 2 (711 bp).

These primers are detailed in Appendix F.

To ensure that I remained blinded to avoid any bias the next set of experiments were designed by me but performed by a laboratory colleague, Dr Li Yan. I provided the extracted DNA, diluted to 10G for 14 individuals – five who had p.L264R mutation identified using the ion torrent, seven who had been identified as wild type for p.L164R and one which was borderline. Dr Yan was not aware which samples were which and all experimental steps were completed in exactly the same manner for the six samples.

The first round of standard PCR was optimised by Dr Li Yan using the primers I designed with an annealing temperature of 55°C and melting temperature of 76°C. The product was then run on an electrophoresis gel, the bands cut and extracted as detailed in 4.3.5 - Extraction and Purification.

This extracted amplified product was then processed in two separate real time PCR experiments using both the ‘short’ primers and the ‘long’ primers in round 2. Short Product PCR was optimised with an

annealing temperature of 50°C and a melting temperature of 79°C. The Long Product PCR was optimised with an annealing temperature of 50°C and a melting temperature of 76°C.

The resultant real-time PCR results confirmed that the mutation specific primers were able to detect the mutation in the three known mutant samples. The ratio of the cycle thresholds for wild type and mutant primers was more clearly demonstrated when the amplified product from round 1 had undergone PCR with the 'short' product primers in round 2. Accordingly we were now able to perform a simple two-step analysis using this technique on DNA extracted from the remaining samples which had not been analysed using the ion torrent.

The ARMS PCR was conducted three times for each of the Ion Torrent samples using both the wild type and mutation specific primers. The cycle threshold values for each PCR were then averaged and plotted on a scatter graph with a line of best fit calculated where more mutant values are below the gradient than above but which precludes the most wild type samples. The gradient of this line was given as +0.5318, Figure 44.

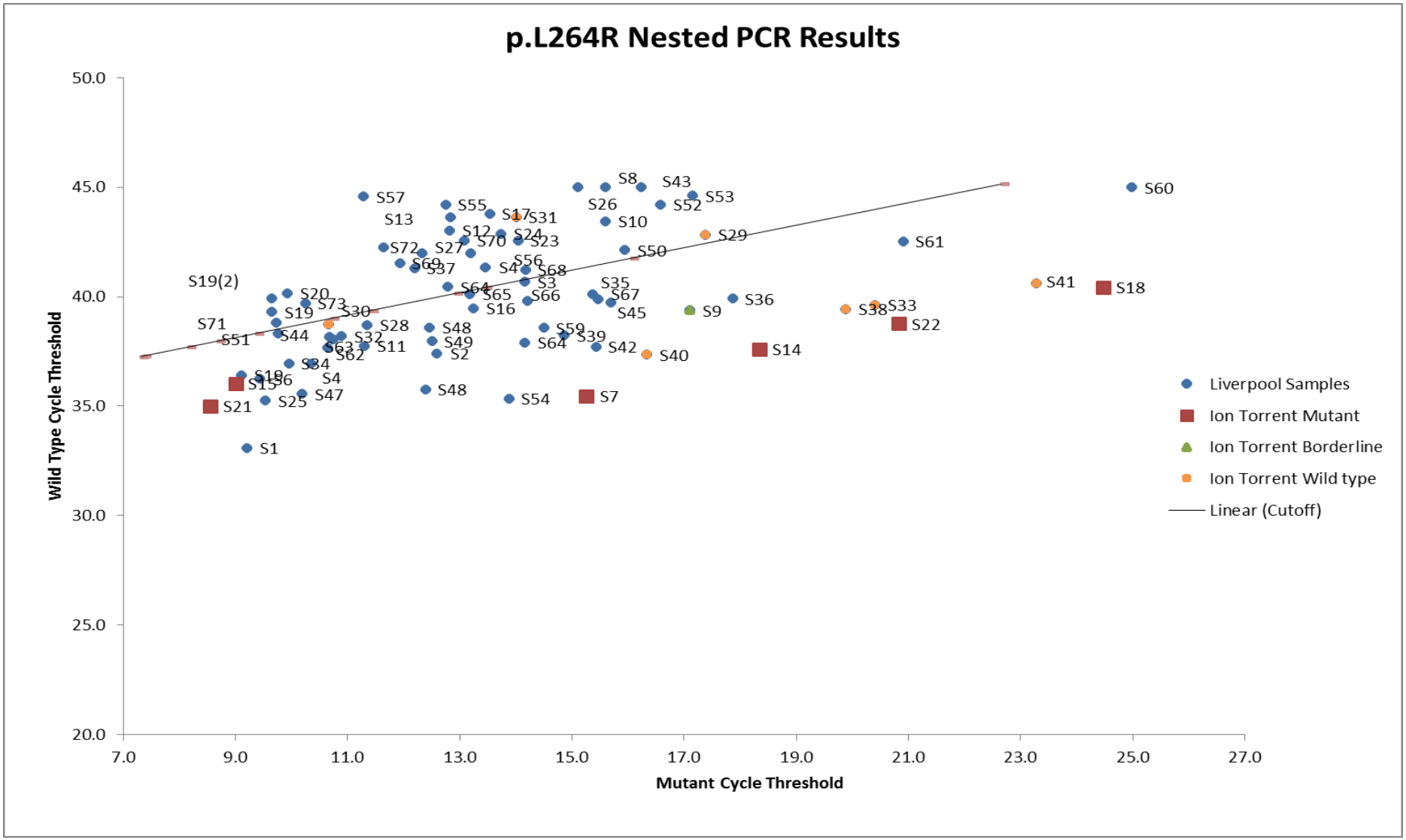


Figure 44: Analysis of wild type and mutant cycle thresholds for validation comparing unknown 'Liverpool' samples with Ion Torrent.

Using this value a receiver-operator curve (ROC) was generated by calculating sensitivity and 1-specificity for each threshold value generated by ARMS and extrapolated using the following formula:

W/T Ct x 0.53185 x *c*

Where '*c*' was given as Ct from 40 – 20 in values decreasing by 0.5. 40, 39.5, 39 etc… The resultant value was then subtracted from the ARMS generated mutant cycle threshold (Mt Ct). If the value was negative then the sample was called MUTANT. If positive (i.e calculated W/T Ct < Mut Ct) then the sample was called WILD TYPE. For each resultant value sensitivity and specificity could be calculated using the descriptors:

Mutant + Dead = True Positive (TP)

Mutant + Alive = False Positive (FP)

Wild Type + Dead = False Negative (FN)

Wild Type + Alive = True Negative (TN)

Sensitivity and Specificity can be readily calculated using:

Sensitivity = TP / (TP+FN)

Sensitivity = TN / (TN+FN)

The resultant data were plotted to create a ROC using SPSS (Figure 45) with an area under the curve calculated at 0.79. From this curve the optimum W/T cycle threshold can be calculated as the point which is maximally sensitive and specific (calculated to lie between 0.66 – 0.83 sensitivity and 0.61 – 0.82 specificity). This correlates to a W/T Ct of 33.5 cycles.

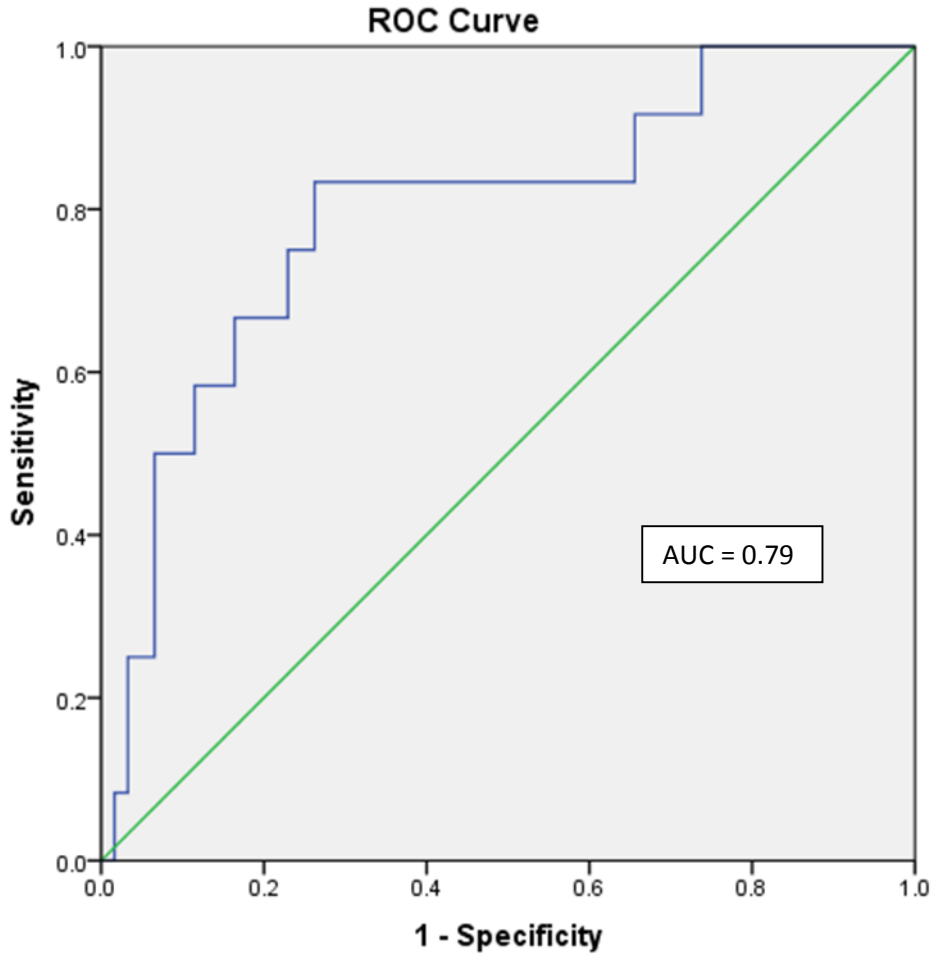


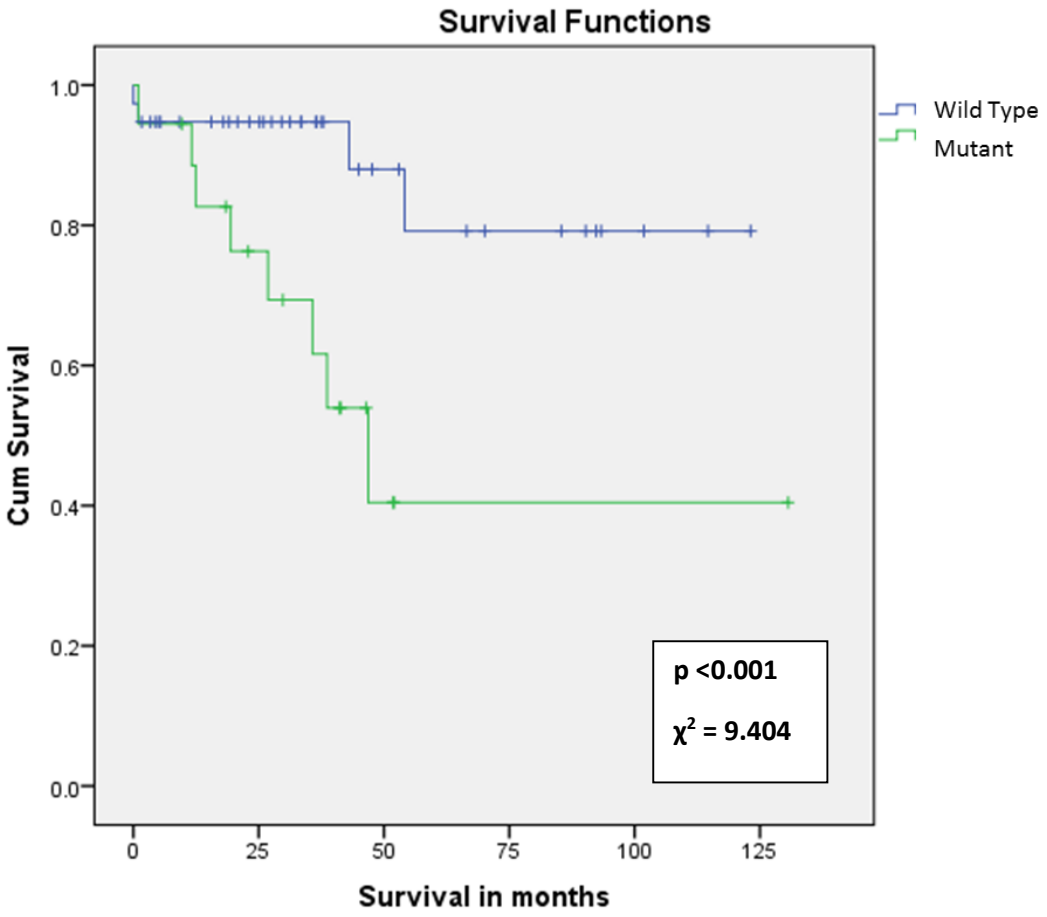
Figure 45: ROC analysis for validation data.

The final formula is then described as:

IF Mut Ct < (W/T Ct x 0.5138) + 33.5 = Mutant Sample

IF Mut Ct > (W/T Ct x 0.5138) + 33.5 = Wild Type Sample.

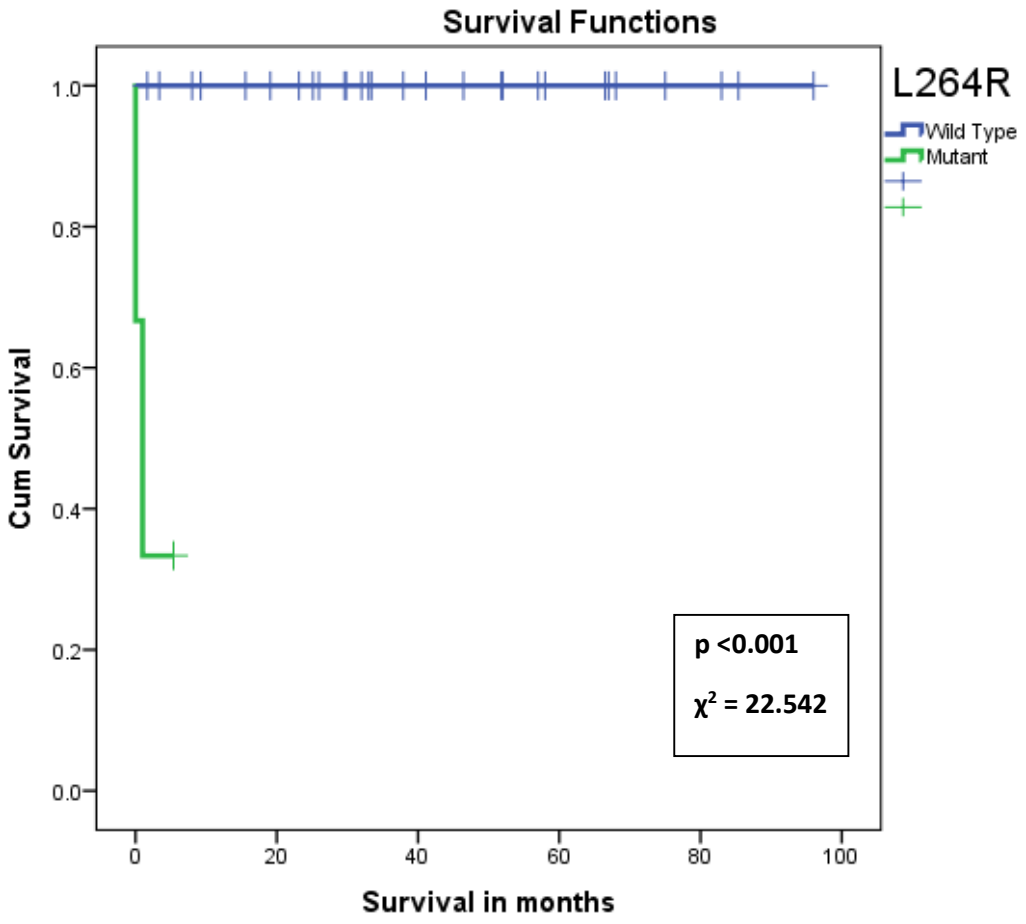
Using this formula applied to the original average Ct readings for each sample they have been designated as mutant or wild type and compared against each other for survival using the Kaplan-Meier Method (Figure 46). As can be seen, there is still a significant difference in survival between those individuals described as mutant using mutation specific PCR and those described as wild type, p=0.005.



	0	25	50	75	100	125
Wild Type	43	28	12	7	3	0
Mutant	13	8	2	1	1	1

Figure 46: Kaplan-Meir analysis of survival for PCR detected p.L264R mutations among all Heidelberg samples.

Following from this, and as a form of validation, I have produced a separate Kaplan-Meir analysis comparing only those samples which were not included in the original Ion Torrent cohort. As can be seen from Figure 47 a further three p.L264R mutations were identified, of which 2 died.



Wild Type	27	22	13	7	3	0
Mutant	3	0	0	0	0	0

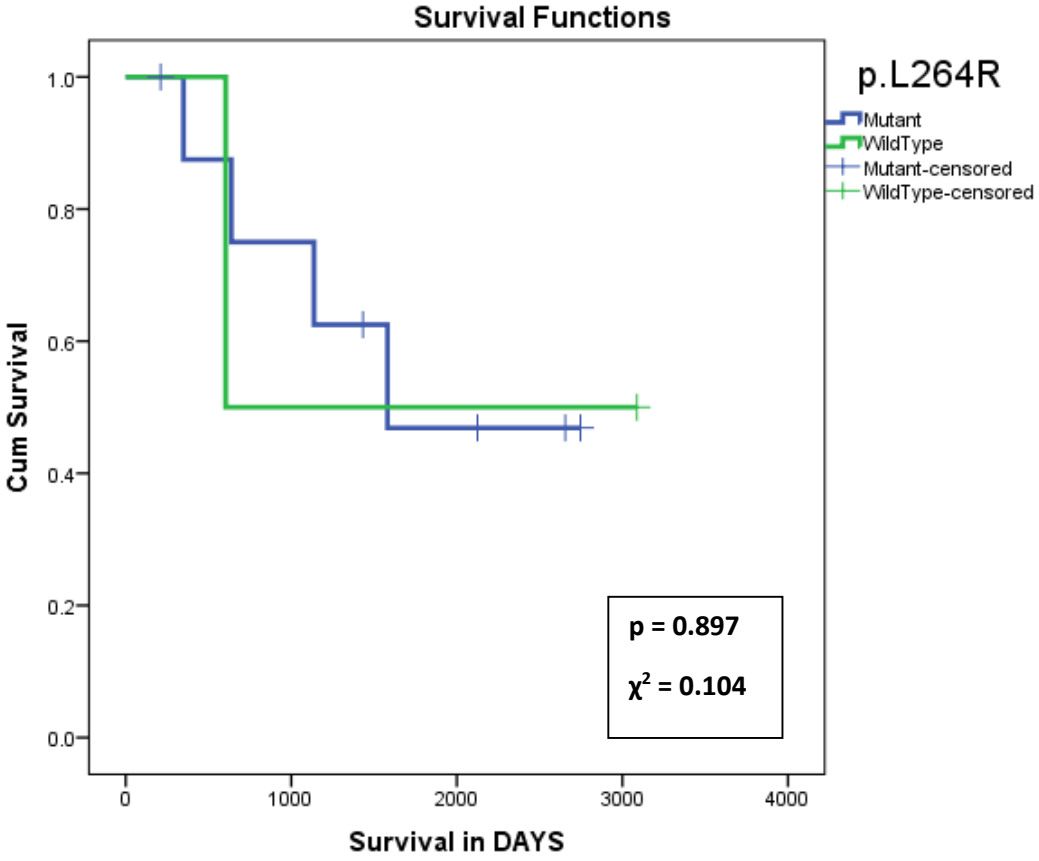
Figure 47: Kaplan-Meir analysis of survival for PCR detected p.L264R mutations among all Heidelberg samples.

Liverpool data

Finally, work was undertaken to perform these mutation specific PCR experiments upon historically resected samples of IPMN from the Liverpool Pancreas Biomedical Research Unit biobank (between 2004 and 2008) which have been stored at -150°C.

This analysis was once again performed in a blinded fashion with the lab work being undertaken by a post-doctoral researcher who was blinded to the histological and survival data. Seven samples were identified, 4 of which were IPMN with concurrent cancer (IPMC) and 3 which were benign IPMN. Using

the methods and formula previously described these samples were analysed to identify their p.L264R mutational status. Only two samples were found to be Wild Type, both of these were found in histologically identified malignant IPMN. The numbers are obviously small and survival analyses does not reveal any significance between those mutant or wild type samples (p=0.897), Figure 48.



	0	1000	2000	3000
Wild Type	2	1	1	0
Mutant	5	3	0	0

Figure 48: Kaplan-Meier survival analyses for p.L264R mutation among 7 Liverpool samples.

Clearly greater numbers are needed and this is work is being undertaken at the time of writing this thesis. The one point of potential significance is that the only sample which was histologically malignant and yet survived was **wild type** for p.L264R. **All** malignant IPMN which were mutant for p.L264R died within 5 years of their resection.

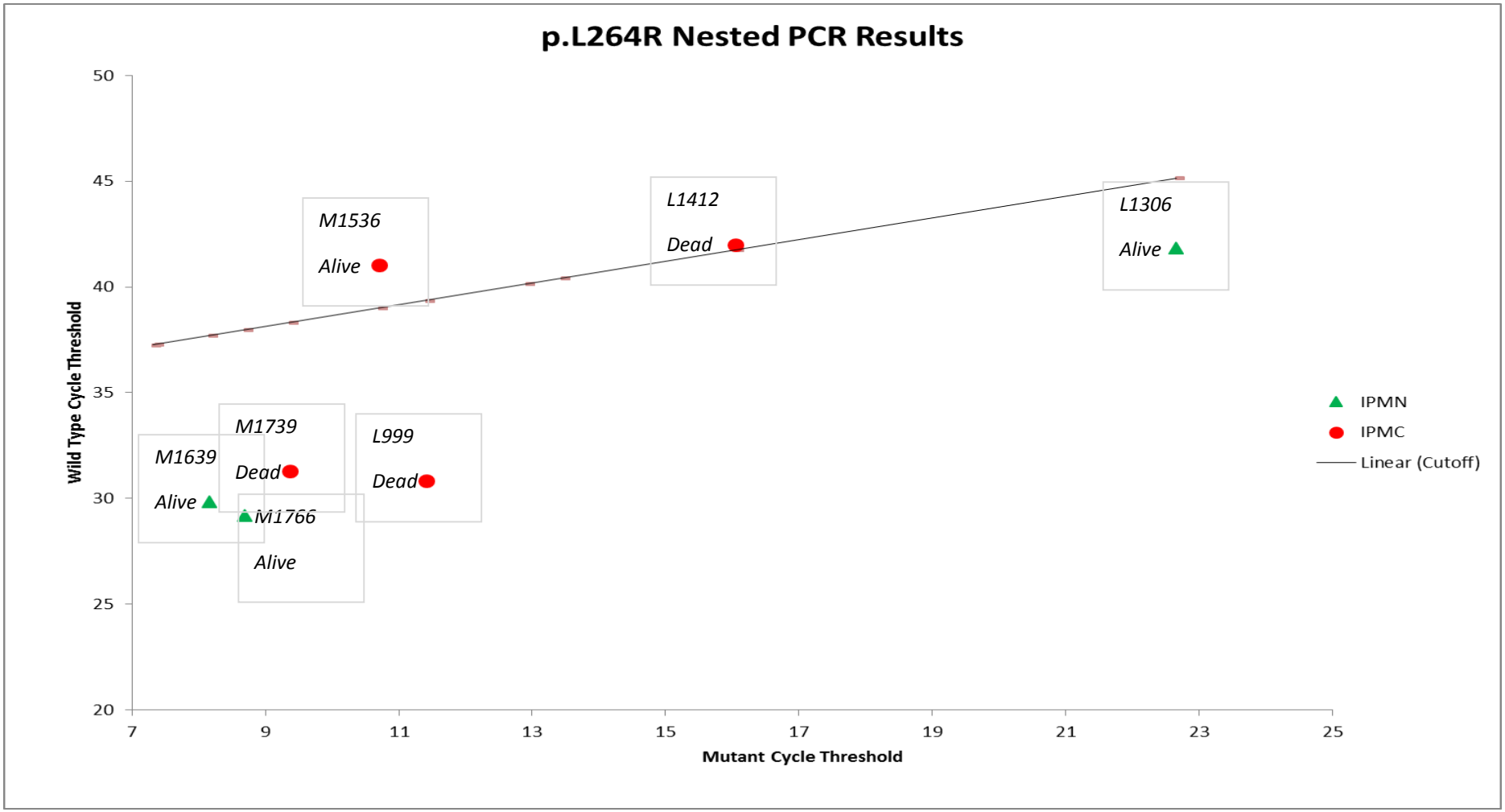


Figure 48: Nested PCR results for Liverpool samples using previously defined cut-off.

Chapter 6
CONCLUSIONS

6 - Conclusions

1) p.L264R in tissue predicts survival ($p<0.005$).
2) Those malignant IPMN which did not have p.L264R were less likely to have nodal spread suggesting that whilst p.L264R is not the trigger for malignancy it may facilitate lymphatic spread and could be a target for palliative or adjuvant chemotherapy.
3) The exact role of p.L264R remains unclear, it is likely to promote binding to MDM2.
4) Limiting dilution is a successful method of removing replicated PCR error from samples undergoing whole genome sequencing.

Chapter 7
DISCUSSION

7 - Discussion

General

Cystic lesions of the pancreas are an increasing phenomenon – it's likely that their incidence is unchanged but that their rate of detection improves with more sophisticated cross-sectional imaging.[100, 102, 272-276] In addition more and more people are undergoing such imaging for a variety of diagnostic reasons and so the incidental finding of IPMNs is set to increase.[31, 277] In one institution in the USA, 356 such lesions were detected over 7 years – the most frequent indications for CT scan were genitourinary complaints and chest pain.[32]

There are numerous current published guidelines for the management of such lesions but pancreatic specialists are still unable to determine with any degree of confidence which of these pose a significant risk to the patient and which can be safely observed or even discharged from 'active' follow-up.[31, 115, 148, 277-285]

As demonstrated, a policy of resection of all such lesions is not feasible given the volume of IPMN detected but also would create levels of morbidity and mortality which are quite unacceptable when taken in the context of the likelihood of malignant progression.[95, 286-291]

Ideally a form of functional imaging, such as PET-CT, would be the best way of assessing the malignant potential of individual IPMN, such trials are underway, and in the future they may well play an important role in the algorithm for the management of incidental IPMN.[292-295] At present however each pancreatic unit relies on their own interpretation of the Sendai criteria resulting in different rates of resection for both malignant and benign IPMN. New work suggests that there may be benefit in the addition of a secretin enhanced MRI in the determination of small BD-IPMN.[296]

When imaging cannot reliably reveal the information required to make a clinical decision further, invasive investigations must be considered. Clearly the role of serum CA19-9 has been proven in pancreatic malignancy, there are of course certain exceptions such as those who carry the Lewis

antibody, but even this stalwart of pancreatic malignancy is proving contentious as a marker for IPMN.[297-299] Further serum markers, most frequently a CEA level of >110ng/mL, have been suggested as predictors of malignancy.[299, 300]

Pancreatic juice (usually obtained at ERCP) has been shown to contain markers that can indicate early pancreatic cancer, until recently it formed the basis of screening for the EUROPAC study. The problem with this approach is the significant risk of harm (either PEP or perforation) which is incurred by virtue of the procedure.[249]

The Goggins' group has shown that pancreatic juice can be reliably collected from within the duodenum of an individual thus negating the risk associated with ERCP.[236, 301] The controversy remains as to the effect of duodenal contamination with both bacterial and ingested DNA, however the very fact that pancreatic DNA can be separated suggests that future collection of pancreatic juice may well be possible with minimal risk to the patient. By definition, whatever the sub-type of IPMN there will always be communication with the main pancreatic duct thus there will be DNA material from the IPMN within the pancreatic juice. There is, of course, the possibility that the detected DNA may well be a product of 'natural' apoptosis of damaged cells which have sloughed off into the main pancreatic duct. This may carry mutation resulting in a false positive finding which would be difficult to avoid.

A more direct approach to obtaining cystic fluid may be via an endoscopic fine-needle aspiration (EUS-FNA). This has been shown in the past to be roughly 70% accurate for the diagnosis of IPMN but as a technique it is still not advocated in the current (2012) Sendai criteria for the management of side branch IPMN.[104, 302] The cytological benefit of obtaining this fluid is based purely in the detection of atypical or malignant cells, since on the morphological characteristics alone it is impossible to distinguish between IPMN and MCN.[303-305] Work is currently underway at the University of Pittsburgh to identify molecular markers which may herald malignancy within cyst fluid but to date, other than association between cyst size and *KRAS* there has been no breakthrough.[62, 306, 307]

This study has shown that the detection of p.L264R **in tissue** is associated with poor survival, it would follow therefore that if this mutation were to be detected in the pancreatic juice of an individual with an IPMN that their IPMN has undergone malignant transformation and the benefit of resection is likely to be minimal or, more optimistically, that the surgery was too late in the collected samples – so equivalent patients should be operated on earlier.

The question of clinical application may therefore be posited away from diagnosis and rather to **prognosis**. If a resected IPMN is subsequently found to be p.L264R mutant this information, in conjunction with TNM staging may be evidence enough to confirm the need for adjuvant chemotherapy.

MUC Status

Mucin protein staining, particularaly MUC1, has been variously associated with a good prognosis in gastric and pancreatic ductal cancer;[308, 309] but also a poor prognosis in high grade lymphoma;[310] renal cell carcinoma;[311] breast cancer;[312] ovarian cancer;[313] cholangiocarcinoma;[314] and colorectal cancer associated with HNPCC.[315]

MUC1 has been implicated in the devlopment of PanIN lesions and has also been shown to be associated with epidermal growth factor receptor tyrosine kinases, therefore postulated to promote cell adhesion and possibly stimulate metastases.[316] Among the 21 samples which were stained for MUC1 there was no discernable correlation between tumour status or metastases, similiarily there is probably no significant correlation between the presence of MUC1 and whether or not the tumour is recurrent as only two samples within the cohort were recurrent tumours.

MUC2 has also been shown to be associated with positive survival outcomes in gastric adenocarcinoma and has been shown to differentiate between mucinous and tubular subtypes of colorectal carcinoma.[317, 318] Interestingly MUC2 is the only MUC protein to have been shown to have discernable differences in expression depending on an individuals race.[319]

It is likley that the gender bias displayed in MUC 2 and MUC 6 may be artefactual and represents the danger of multiple testing though there was no obvious gender bias in MUC 5 samples.

In this work MUC 6 is perhaps associated with lower grade dysplasia (p=0.05) but I would only have expected MUC 6 to be present in the oncocytic subtype. In my cohort MUC 6 was positive in 9, including the oncocytic sample (CYST023) thus suggesting that 8 of the remainig samples were 'mixed' IPMN with an element of oncoytic epithelium present. This raises the question of whether the conventional wisdom that IPMN should be graded in line with the most 'sinister' subtype (gastric>intestinal>pancreatobiliary>oncocytic) holds true in all cases as this cohort includes 8 cases which should therefore be classed as oncocytic.

Samples which were MUC 2 positive did have a tendency towards significance for survival (p=0.054) which is difficult to explain in the context of a lack of association between grade of dysplasia or TNM status.

In essence, within the confines of this work, the MUC staining was more useful for delineating the epithelial sub-type of IPMN rather than displaying any significant prognostic associations.

Mutational Analyses

The initial assessment of those individuals with p.M160L mutation suggested a trend towards increasing tumour status (Table 30), however when tumour status is defined as high (>T2) or low (≤T2) there is no difference (4/13 vs. 5/14, p=0.78).

My research suggested a relationship between p.I251L and smoking (8 out of 14 current or ex-smokers were found to have the mutation compared to 4/27 non-smokers, p=0.06). In 2002 researchers in California confirmed that there are a greater percentage of p53 mutations in smokers lung cancer (30%) versus non-smokers lung cancer (12%).[320] These mutations were clustered between codons 254-249 however. Furthermore, it has long been established that the most common p53 mutations

associated with smoking are G>T transversions, whereas p.I251L is the opposite transversion (A>C).[210] This mutation has been previously reported in stomach and bladder cancers.[321] In gastric cancer, research on smoking mediated p53 mutational status has focussed on codon 72 and the interaction with *Helicobacter pylori*.[322] There is insufficient evidence, due to the small numbers involved in this sample, to accurately assess the likelihood of smoking being a causative factor in IPMN development, this is born out in a recent large scale retrospective analysis of 446 patients.[323] Certainly we have not demonstrated an association between smoking and maligant progression, nor indeed survival.

An association has been suggested between diabetes and the p.E271G mutation in this work. Among 8 diabetics who were undergoing treatment (either with insulin therapy or oral anti-hyperglycaemic agents) 4 (50%) were found to express the mutation compared to 5 out of 30 (17%) non-diabetics, p=0.048). Little has been proven with regards to the relatonship between p53 and diabetes, research conducted in obese mice have uncovered a p53-dependent senescence response in fat cells; this response ultimately engendered insulin resistance.[172, 198] Diabetes was not independently shown to be a significant co-factor for survival, grade of dysplasia or severity of tumour (TNM stage).

Mutations

The following data has been collated from the IARC p53 databse (http://p53.iarc.fr/*TP53*GeneVariations.aspx).

p.K132Q (c.394A>C)

On the basis of yeast functional assays this is a loss of function mutation as with the promoters of p53R2, NOXA, GAD45 and AIP this mutation gives less than 10% of wild type activity.

This mutation was first described in a B-cell lymphoma and has been described as a somatic mutation in pancreatic adenocarcinoma on one occasion, the mutation has been shown to be present in the pancreatic cancer cell line PSN1.[324,325] Of the 4 occurrences in my Ion Torrent samples 3 were IPMC and 1 IPMN. One case died at 26.86 months. Two of the IPMN were intestinal subtype and one gastric. The IPMC was a mucinous adenocarcinoma.

Unpublished work at the University of Florida is attempting to prove the hypothesis that p.K164 is essential for transferring p53 out of the nucleus and into the cytoplasm of the cell (http://cur.aa.ufl.edu/Data/Sites/5/media/symposium2013/symposium-book-2013-final.pdf).

p.M160L (c.478A>C)

On the basis of yeast functional assays this is not a loss of function mutation as with the promoters of p53R2 and NOXA it results in greater than wild type activity and for the promoters of GAD45 and AIP greater than 70% activity.

This mutation was first described in a MALT (Mucosal Associated Lymphoid Tissue)-lymphoma and has also been described as a somatic mutation in cholangiocarcinoma but is not known to be present in any cell lines.[326-328] Of the 10 occurrences in my sample 8 were IPMC, 1 PDAC and only one IPMN. Five of the cases died with a median survival of 35.73 months. It is thought that p.M160L may be a mutation which adversely affects the DNA binding domain of p53 by altering the β-sheet in the core of the domain.[329]

p.K164R (c.491A>G)

On the basis of yeast functional assays this is not a loss of function mutation as with the promoters of p53R2 and NOXA it results in greater than wild type activity and for the promoters of GAD45 and AIP greater than 50% activity.

This mutation was first reported in colorectal cancer and has been described in pancreatic cancer twice and in one case of liver-fluke associated cholangiocarcinoma.[330-334] Of the 9 occurrences in my sample all were malignant IPMN (IPMC), three of whom died with a median survival of 43 months. Interestingly all three of those who died also carried the p.L264R mutation and all of those who survived were wild type for p.L164R. Work in 2008 suggested that mutation at codon 164 may lead to loss of acetylation which will reduce the ability of p53 to activate p.21 and also blocks the interaction of p53 and MDM2.[335] As a consequence the p53 will be activated regardless of its phosphorylation status.

p.K164T (c.491A>C)

On the basis of yeast functional assays this is not a loss of function mutation as with the promoters of p53R2 and NOXA it results in greater than wild type activity and for the promoters of GAD45 and AIP greater than 40% activity.

This mutation was first reported in an ovarian cancer and has never been described in pancreatic or biliary cancers.[336] Of the 3 occurrences in my sample all were malignant mixed IPMN 1 died at 38.6 months. Little is understood about this mutation and there are currently no published hypotheses for the possible action on the p53 molecule.

p.I251L (c.751A>C)

On the basis of yeast functional assays this is a loss of function mutation as with the promoters of NOXA, GAD45 and AIP it gives less than 10% of wild type activity and with p53R2 promoter only 25% activity.

This mutation was first reported in a gastric cancer but has never been described in pancreatic or biliary cancer.[337] This mutation is known to be present in three separate stomach cancer cell-lines: MK28, MK7 and MK24.[338, 339] Amongst the 10 occurrences in my sample, 9 were IPMC and one benign. One patient died at 35 months and another died of post-operative complications. No specific work has been published which looks at this mutation, however the I251 position is known to be an area of hydrophobic binding for the molecule RelA-associated inhibitor which has been shown to interact with p53 by inhibiting the DNA-binding activity.[340]

p.L264R (c.791T>G)

On the basis of yeast functional assays this is not a loss of function mutation as with the promoters of p53R2, NOXA, GAD45 and AIP it gives greater than 10% of wild type activity.

First described in colorectal cancer, this mutation has been identified to have occurred in pancreatic cancer on one occasion and is also known to be present in the cell line TK-10.[139, 341, 342] As described in chapter 5, this is the only mutation which demonstrated a significant association with survival. There have been so specific publications, to date, which have looked at the function of p.L264R

specifically. A study of ribozyme mediated modification of p53 mRNA in lung cancer demonstrated that even when codon 264 was targeted there was no suppression of cell growth.[343]

p.L264 forms part of the linker which bridges the S9–S10 β-sheets of p53 and mutation of this site has been shown to give increased MDM2-dependent ubiquitination of p53.[344]

p.E271G (c.812A>G)

On the basis of yeast functional assays this is not a loss of function mutation as with the promoters of p53R2, NOXA, GAD45 and AIP it gives greater than 70% activity.

This mutation was first described in a mouth cancer and has never previously been reported in pancreatic or biliary cancer.[345] Of the 9 cases in my sample p.E271G never occurred as a sole mutation and 8 of the cases were IPMC with one IPMN. Three cases died with a median survival of 26.8 months. E271 is known to be one the two important glutamate residues which bind to damaged DNA via PARP-1 leading to inhibited nuclear export of p53 and apoptosis.[329]

p.R273H (c.818G>A)

On the basis of yeast functional assays this is a loss of function mutation as with the promoters of NOXA, GAD45 and AIP promoters it gives less than 10% activity and for p53R2 just 16% activity.

The most common mutation described in p53, R273H was first described in lung cancer in 1990.[346] It has since been discovered to occur in 50 different cancers as well as 80 cell lines and 54 reported germline mutations are available in the literature. R273H has been identified to have occurred in 14 different pancreatic tumours.[333, 347-359] Amongst the 4 occurrences of this mutation in my sample, 2 were benign IPMN who both survived and 2 were IPMC and who both died with a median survival of 34.95 months. R273 mutations are known to adversely affect p53 function in two major ways: the mutation change the protein confirmation which prevents the formation of the functional tetramer and;[360] asserts a 'transdominance' effect which promotes propagation of the mutant p53 in favour of the wild type protein.[361]

Limitations

The number of samples in this work is small, yet these data are compelling. The concern that there may be a geographical bias to the development of this somatic mutation is negated by the detection of p.L264R in samples obtained from Liverpool – with similar survival outcomes. Whether or not this mutation is caused by specific environmental stresses is still open – though we have looked at diabetes, alcohol and smoking insofar as the data we hold has allowed.

If such a mutation can be demonstrated to be a product of or accentuated by external factors then where possible advice should be given to avoid such precursors.

Immunohistochemistry confirmed the subtype of IPMN (gastric, intestinal, oncocytic, pancreatobiliary) used within the study but did not bear any correlation with survival, nor the expression of p.L264R.

It is apparent from an assessment of the median depth of reads per exon (Table 27) that Exon 6 had a relative paucity of reads – this may well be the reason that none of the mutations detected using the whole genome sequence method were located in exon 6. It is already known, however, that mutations are comparatively less common in Exon 6 compared to 5, 7 and 8 as can be seen in Figure 49 taken from the IARC p53 database.

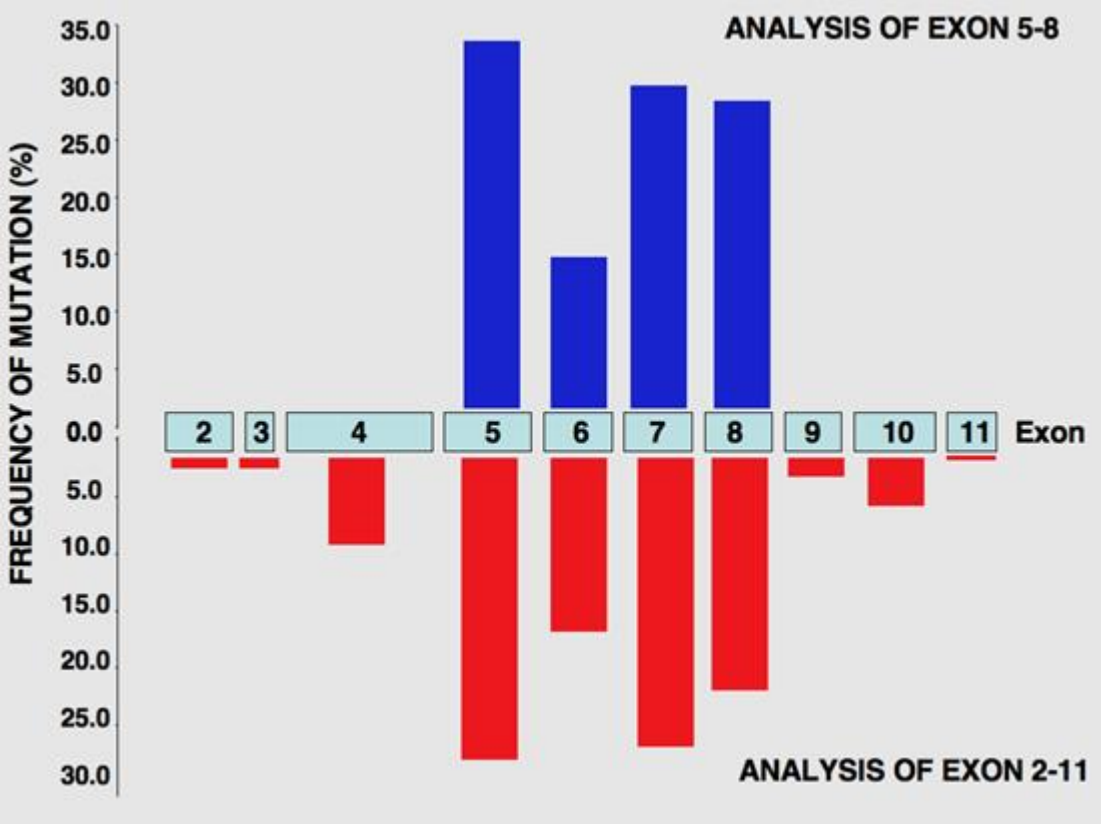


Figure 49: Frequency of mutations of p53 by exon.

Initially I was concerned that my method of 'blind' dissection of the samples provided from Heidelberg would mean that mutational analyses would not reflect the neoplasm present. In fact, as can be seen from the fact that many of the samples processed were felt to represent benign conditions (such as chronic pancreatitis) the fact that p53 mutations were detected in 'normal' paraneoplastic tissue adds weight to the idea of a 'field theory' of mutational change surrounding the initial neoplastic focus.[362] The surrounding cells are harbouring mutations in readiness for malignant transformation before they have been phenotypically altered.

A final point to be made in terms of limitation of this work would be to raise the suggestion that the somatic p.L264R mutation may well be a geographic phenomenon – certainly the results among (an admittedly smaller) Liverpool selection of patients do not bear the same startling significance as the German cohort, although the same trend is seen. I do not think that this diminishes the significance of the work, even if the results are subsequently found to be applicable to German patients there is still the potential for a significant benefit.

Summary

There are relatively few reports of mutations at codon 264 of p53 (45 at the time of writing). However, p.L264R has been seen previously in renal cell cancer and exists in cell line (TK-10) which may provide access to future studies to try and discover the effect of this mutation on the overall function of the p53 protein. The suggestion from the literature is that this mutation may act chiefly to increase binding of p53 to MDM2[344], which combined with its maintained activity in promoting expression of essential genes involved in survival in conditions of stress (p53R2, NOXA, GAD45 and AIP) may imply that the mutation does not prevent the survival promoting functions of p53 but instead attenuates the functions that require high levels of the protein (such as induction of apoptosis). This in turn would imply that p.L264R would be a well-tolerated mutation offering a pre-malignant lesion the opportunity to develop further, with a continuing requirement for other p53 mutations in order for the lesion to finally develop into a carcinoma.

The work of Goggins et.al. at Johns Hopkins has shown that it is possible to detect lesions from pancreatic cysts in duodenal aspirate, indeed in Liverpool our own experience with duodenal aspirate has yielded similar results (p.89).[363] It is feasible, therefore, so suggest that further work could be aimed at detecting p.L264R in duodenal aspirate of individuals with CT proven IPMN to prospectively prove correlation with p.L264R and outcomes.

Whether the future role of p.L264R is destined to be a screening tool, a therapeutic target or a prognostic marker there is sufficient weight of evidence within this thesis to advocate further investigation into the role of thesis SNP in the pathogenesis of IPMN associated pancreatic cancer. I remain firmly committed to my prediction that the cure for pancreatic cancer will be found in epigenetics not the operating theatre. To misquote Paracelsus:

> "There can be no surgeon who is not also a *scientist*".

Chapter 8
REFERENCES

8 - References

1. UK CR. Pancreatic Cancer Incidence Statistics. 2013 17/05/2013 [cited 2014 28/05/2014]; Available from: http://www.cancerresearchuk.org/cancer-info/cancerstats/types/pancreas/incidence/
2. Mayor S. One year cancer survival rates in England remain poor despite national strategies, report finds. BMJ. 2011; **342**: d1374.
3. UK CR. Pancreatic Cancer Survival Statistics. 2012 03/09/2012 [cited 2014 28/05/2014]; Available from: http://www.cancerresearchuk.org/cancer-info/cancerstats/types/pancreas/survival/pancreatic-cancer-survival-statistics#source1
4. Mishra MV, Keith SW, Shen X, Ad VB, Champ CE, Biswas T. Primary Pancreatic Lymphoma: A Population-based Analysis Using the SEER Program. American journal of clinical oncology. 2011.
5. Murakami H, Tsuchiya K, Nishimura H, Ushikubi F, Atsumi K, Seki M, et al. [Hereditary pancreatitis]. Nihon Shokakibyo Gakkai zasshi = The Japanese journal of gastro-enterology. 1983; **80**(4): 1031-5.
6. Starling N, Cunningham D. Survival from cancer of the pancreas in England and Wales up to 2001. British journal of cancer. 2008; **99 Suppl 1**: S24-5.
7. Woo SM, Ryu JK, Lee SH, Yoo JW, Park JK, Kim YT, et al. Survival and prognosis of invasive intraductal papillary mucinous neoplasms of the pancreas: comparison with pancreatic ductal adenocarcinoma. Pancreas. 2008; **36**(1): 50-5.
8. Niedergethmann M, Grutzmann R, Hildenbrand R, Dittert D, Aramin N, Franz M, et al. Outcome of invasive and noninvasive intraductal papillary-mucinous neoplasms of the pancreas (IPMN): a 10-year experience. World journal of surgery. 2008; **32**(10): 2253-60.
9. Goldstein J, Benharroch D, Sion-Vardy N, Arish A, Levy I, Maor E. Solid cystic and papillary tumor of the pancreas with oncocytic differentiation. Journal of surgical oncology. 1994; **56**(1): 63-7.
10. Tajiri H, Yoshimori M, Nakamura K, Ozaki H, Taylor WF, Go VL. [Clinicopathological study on carcinoma of the pancreas operated on at the Mayo Clinic and the National Cancer Center Hospital--a comparison of the diagnostic process in both institutions and an analysis of survival rates]. Nihon Gan Chiryo Gakkai shi. 1987; **22**(3): 566-72.
11. Maire F, Sauvanet A, Couvelard A, Rebours V, Vullierme MP, Lebtahi R, et al. Recurrence after surgical resection of gastrinoma: who, when, where and why? European journal of gastroenterology & hepatology. 2012; **24**(4): 368-74.
12. Grygiel K, Szmidt J, Jelenska M, Pawlak K. Surgical treatment of hyperinsulinism during the course of pancreatic cancer (insulinoma) - one center experience. Polski przeglad chirurgiczny. 2012; **84**(1): 31-6.
13. Eldor R, Glaser B, Fraenkel M, Doviner V, Salmon A, Gross DJ. Glucagonoma and the glucagonoma syndrome - cumulative experience with an elusive endocrine tumour. Clinical endocrinology. 2011; **74**(5): 593-8.
14. Elshafie O, Grant C, Al-Hamdani A, Jain R, Woodhouse N. VIPoma Crisis: Immediate and life saving reduction of massive stool volumes on starting treatment with octreotide. Sultan Qaboos University medical journal. 2011; **11**(1): 104-7.
15. Evans J, Chapple A, Salisbury H, Corrie P, Ziebland S. "It can't be very important because it comes and goes"--patients' accounts of intermittent symptoms preceding a pancreatic cancer diagnosis: a qualitative study. BMJ open. 2014; **4**(2): e004215.
16. Porta M, Fabregat X, Malats N, Guarner L, Carrato A, de Miguel A, et al. Exocrine pancreatic cancer: symptoms at presentation and their relation to tumour site and stage. Clinical & translational oncology : official publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico. 2005; **7**(5): 189-97.

17. Gobbi PG, Bergonzi M, Comelli M, Villano L, Pozzoli D, Vanoli A, et al. The prognostic role of time to diagnosis and presenting symptoms in patients with pancreatic cancer. Cancer epidemiology. 2013; **37**(2): 186-90.
18. Silverman DT. Risk factors for pancreatic cancer: a case-control study based on direct interviews. Teratogenesis, carcinogenesis, and mutagenesis. 2001; **21**(1): 7-25.
19. Koprowski H, Herlyn M, Steplewski Z, Sears HF. Specific antigen in serum of patients with colon carcinoma. Science. 1981; **212**(4490): 53-5.
20. Goonetilleke KS, Siriwardena AK. Systematic review of carbohydrate antigen (CA 19-9) as a biochemical marker in the diagnosis of pancreatic cancer. European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology. 2007; **33**(3): 266-70.
21. Gui JC, Yan WL, Liu XD. CA19-9 and CA242 as tumor markers for the diagnosis of pancreatic cancer: a meta-analysis. Clinical and experimental medicine. 2014; **14**(2): 225-33.
22. Narimatsu H, Iwasaki H, Nakayama F, Ikehara Y, Kudo T, Nishihara S, et al. Lewis and secretor gene dosages affect CA19-9 and DU-PAN-2 serum levels in normal individuals and colorectal cancer patients. Cancer research. 1998; **58**(3): 512-8.
23. Tempero MA, Uchida E, Takasaki H, Burnett DA, Steplewski Z, Pour PM. Relationship of carbohydrate antigen 19-9 and Lewis antigens in pancreatic cancer. Cancer research. 1987; **47**(20): 5501-3.
24. Yeo C, Yeo, TP, Hruban, RH, et. al., editor. Cancer of the Pancreas. 7th ed. Philadelphia: Lippincott Williams & Wilkins; 2004.
25. Procacci C, Graziani R, Bicego E, Bergamo-Andreis IA, Mainardi P, Zamboni G, et al. Intraductal mucin-producing tumors of the pancreas: imaging findings. Radiology. 1996; **198**(1): 249-57.
26. Megibow AJ, Lombardo FP, Guarise A, Carbognin G, Scholes J, Rofsky NM, et al. Cystic pancreatic masses: cross-sectional imaging observations and serial follow-up. Abdominal imaging. 2001; **26**(6): 640-7.
27. Long EE, Van Dam J, Weinstein S, Jeffrey B, Desser T, Norton JA. Computed tomography, endoscopic, laparoscopic, and intra-operative sonography for assessing resectability of pancreatic cancer. Surgical oncology. 2005; **14**(2): 105-13.
28. Vargas R, Nino-Murcia M, Trueblood W, Jeffrey RB, Jr. MDCT in Pancreatic adenocarcinoma: prediction of vascular invasion and resectability using a multiphasic technique with curved planar reformations. AJR American journal of roentgenology. 2004; **182**(2): 419-25.
29. Soriano A, Castells A, Ayuso C, Ayuso JR, de Caralt MT, Gines MA, et al. Preoperative staging and tumor resectability assessment of pancreatic cancer: prospective study comparing endoscopic ultrasonography, helical computed tomography, magnetic resonance imaging, and angiography. The American journal of gastroenterology. 2004; **99**(3): 492-501.
30. Winter JM, Cameron JL, Lillemoe KD, Campbell KA, Chang D, Riall TS, et al. Periampullary and pancreatic incidentaloma: a single institution's experience with an increasingly common diagnosis. Annals of surgery. 2006; **243**(5): 673-80; discussion 80-3.
31. Sachs T, Pratt WB, Callery MP, Vollmer CM, Jr. The incidental asymptomatic pancreatic lesion: nuisance or threat? Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract. 2009; **13**(3): 405-15.
32. Bruzoni M, Johnston E, Sasson AR. Pancreatic incidentalomas: clinical and pathologic spectrum. American journal of surgery. 2008; **195**(3): 329-32; discussion 32.
33. Ghaneh P, Costello E, Neoptolemos JP. Biology and management of pancreatic cancer. Gut. 2007; **56**(8): 1134-52.
34. Shore S, Raraty MG, Ghaneh P, Neoptolemos JP. Review article: chemotherapy for pancreatic cancer. Alimentary pharmacology & therapeutics. 2003; **18**(11-12): 1049-69.
35. Neoptolemos JP, Cunningham D, Friess H, Bassi C, Stocken DD, Tait DM, et al. Adjuvant therapy in pancreatic cancer: historical and current perspectives. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2003; **14**(5): 675-92.

36. Sultana A, Cox T, Ghaneh P, Neoptolemos JP. Adjuvant therapy for pancreatic cancer. Recent results in cancer research Fortschritte der Krebsforschung Progres dans les recherches sur le cancer. 2012; **196**: 65-88.
37. Sultana A, Tudur Smith C, Cunningham D, Starling N, Tait D, Neoptolemos JP, et al. Systematic review, including meta-analyses, on the management of locally advanced pancreatic cancer using radiation/combined modality therapy. British journal of cancer. 2007; **96**(8): 1183-90.
38. Rose JB, Rocha FG, Alseidi A, Biehl T, Moonka R, Ryan JA, et al. Extended neoadjuvant chemotherapy for borderline resectable pancreatic cancer demonstrates promising postoperative outcomes and survival. Annals of surgical oncology. 2014; **21**(5): 1530-7.
39. Chen KT, Devarajan K, Milestone BN, Cooper HS, Denlinger C, Cohen SJ, et al. Neoadjuvant chemoradiation and duration of chemotherapy before surgical resection for pancreatic cancer: does time interval between radiotherapy and surgery matter? Annals of surgical oncology. 2014; **21**(2): 662-9.
40. Alexakis N, Halloran C, Raraty M, Ghaneh P, Sutton R, Neoptolemos JP. Current standards of surgery for pancreatic cancer. The British journal of surgery. 2004; **91**(11): 1410-27.
41. Birkmeyer JD, Finlayson SR, Tosteson AN, Sharp SM, Warshaw AL, Fisher ES. Effect of hospital volume on in-hospital mortality with pancreaticoduodenectomy. Surgery. 1999; **125**(3): 250-6.
42. Birkmeyer JD, Siewers AE, Finlayson EV, Stukel TA, Lucas FL, Batista I, et al. Hospital volume and surgical mortality in the United States. The New England journal of medicine. 2002; **346**(15): 1128-37.
43. Birkmeyer JD, Warshaw AL, Finlayson SR, Grove MR, Tosteson AN. Relationship between hospital volume and late survival after pancreaticoduodenectomy. Surgery. 1999; **126**(2): 178-83.
44. Winter JM, Cameron JL, Campbell KA, Arnold MA, Chang DC, Coleman J, et al. 1423 pancreaticoduodenectomies for pancreatic cancer: A single-institution experience. Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract. 2006; **10**(9): 1199-210; discussion 210-1.
45. Chen T, Wang H, Song Y, Li X, Wang J. POSSUM and P-POSSUM as predictors of postoperative morbidity and mortality in patients undergoing hepato-biliary-pancreatic surgery: a meta-analysis. Annals of surgical oncology. 2013; **20**(8): 2501-10.
46. Neoptolemos JP, Stocken DD, Dunn JA, Almond J, Beger HG, Pederzoli P, et al. Influence of resection margins on survival for patients with pancreatic cancer treated by adjuvant chemoradiation and/or chemotherapy in the ESPAC-1 randomized controlled trial. Annals of surgery. 2001; **234**(6): 758-68.
47. Ghaneh P, Neoptolemos JP. Conclusions from the European Study Group for Pancreatic Cancer adjuvant trial of chemoradiotherapy and chemotherapy for pancreatic cancer. Surgical oncology clinics of North America. 2004; **13**(4): 567-87, vii-viii.
48. Oettle H, Post S, Neuhaus P, Gellert K, Langrehr J, Ridwelski K, et al. Adjuvant chemotherapy with gemcitabine vs observation in patients undergoing curative-intent resection of pancreatic cancer: a randomized controlled trial. JAMA : the journal of the American Medical Association. 2007; **297**(3): 267-77.
49. de Castro SM, Houwert JT, Lagarde SM, Reitsma JB, Busch OR, van Gulik TM, et al. Evaluation of POSSUM for patients undergoing pancreatoduodenectomy. World journal of surgery. 2009; **33**(7): 1481-7.
50. Debinska I, Smolinska K, Osiniak J, Paluszkiewicz P. The possum scoring system and complete blood count in the prediction of complications after pancreato-duodenal area resections. Polski przeglad chirurgiczny. 2011; **83**(1): 10-8.
51. Gallacher PJ RN, Duxbury M, et al. The evaluation of the POSSUM scoring system in the prediction of morbidity following pancreaticoduodenectomy. British Journal of Surgery. 2011; **98 (Suppl. 3)**: 44.

52. Khan AW, Shah SR, Agarwal AK, Davidson BR. Evaluation of the POSSUM scoring system for comparative audit in pancreatic surgery. Digestive surgery. 2003; **20**(6): 539-45.

53. Knight BC, Kausar A, Manu M, Ammori BA, Sherlock DJ, O'Reilly DA. Evaluation of surgical outcome scores according to ISGPS definitions in patients undergoing pancreatic resection. Digestive surgery. 2010; **27**(5): 367-74.
54. Pratt W, Joseph S, Callery MP, Vollmer CM, Jr. POSSUM accurately predicts morbidity for pancreatic resection. Surgery. 2008; **143**(1): 8-19.
55. Tamijmarane A, Bhati CS, Mirza DF, Bramhall SR, Mayer DA, Wigmore SJ, et al. Application of Portsmouth modification of physiological and operative severity scoring system for enumeration of morbidity and mortality (P-POSSUM) in pancreatic surgery. World journal of surgical oncology. 2008; **6**: 39.
56. Zhang Y, Fu L, Zhang ZD, Li ZJ, Liu XB, Hu WM, et al. Evaluation of POSSUM in predicting post-operative morbidity in patients undergoing pancreaticoduodenectomy. The Journal of international medical research. 2009; **37**(6): 1859-67.
57. Lahat G, Ben Haim M, Nachmany I, Sever R, Blachar A, Nakache R, et al. Pancreatic incidentalomas: high rate of potentially malignant tumors. Journal of the American College of Surgeons. 2009; **209**(3): 313-9.
58. Rodriguez JR, Salvia R, Crippa S, Warshaw AL, Bassi C, Falconi M, et al. Branch-duct intraductal papillary mucinous neoplasms: observations in 145 patients who underwent resection. Gastroenterology. 2007; **133**(1): 72-9; quiz 309-10.
59. Spinelli KS, Fromwiller TE, Daniel RA, Kiely JM, Nakeeb A, Komorowski RA, et al. Cystic pancreatic neoplasms: observe or operate. Annals of surgery. 2004; **239**(5): 651-7; discussion 7-9.
60. Richards MA. Trends and inequalities in survival for 20 cancers in England and Wales 1986-2001: population-based analyses and clinical commentaries. Foreword. British journal of cancer. 2008; **99 Suppl 1**: S1.
61. Hruban RH, Takaori K, Klimstra DS, Adsay NV, Albores-Saavedra J, Biankin AV, et al. An illustrated consensus on the classification of pancreatic intraepithelial neoplasia and intraductal papillary mucinous neoplasms. The American journal of surgical pathology. 2004; **28**(8): 977-87.
62. Khalid A, Zahid M, Finkelstein SD, LeBlanc JK, Kaushik N, Ahmad N, et al. Pancreatic cyst fluid DNA analysis in evaluating pancreatic cysts: a report of the PANDA study. Gastrointestinal endoscopy. 2009; **69**(6): 1095-102.
63. Sawai H, Okada Y, Funahashi H, Matsuo Y, Tanaka M, Manabe T. Immunohistochemical analysis of molecular biological factors in intraductal papillary-mucinous tumors and mucinous cystic tumors of the pancreas. Scandinavian journal of gastroenterology. 2004; **39**(11): 1159-65.
64. Traverso LW, Moriya T, Hashimoto Y. Intraductal papillary mucinous neoplasms of the pancreas: making a disposition using the natural history. Current gastroenterology reports. 2012; **14**(2): 106-11.
65. Logan SE, Voet RL, Tompkins RK. The malignant potential of mucinous cysts of the pancreas. The Western journal of medicine. 1982; **136**(2): 157-62.
66. Verbeke CS. Intraductal papillary-mucinous neoplasia of the pancreas: Histopathology and molecular biology. World journal of gastrointestinal surgery. 2010; **2**(10): 306-13.
67. Cellier C, Cuillerier E, Palazzo L, Rickaert F, Flejou JF, Napoleon B, et al. Intraductal papillary and mucinous tumors of the pancreas: accuracy of preoperative computed tomography, endoscopic retrograde pancreatography and endoscopic ultrasonography, and long-term outcome in a large surgical series. Gastrointestinal endoscopy. 1998; **47**(1): 42-9.
68. Furukawa T, Kloppel G, Volkan Adsay N, Albores-Saavedra J, Fukushima N, Horii A, et al. Classification of types of intraductal papillary-mucinous neoplasm of the pancreas: a consensus study. Virchows Archiv : an international journal of pathology. 2005; **447**(5): 794-9.
69. Terris B, Dubois S, Buisine MP, Sauvanet A, Ruszniewski P, Aubert JP, et al. Mucin gene expression in intraductal papillary-mucinous pancreatic tumours and related lesions. The Journal of pathology. 2002; **197**(5): 632-7.

70. Furukawa T, Takahashi T, Kobari M, Matsuno S. The mucus-hypersecreting tumor of the pancreas. Development and extension visualized by three-dimensional computerized mapping. Cancer. 1992; **70**(6): 1505-13.
71. Werner J, Fritz S, Buchler MW. Intraductal papillary mucinous neoplasms of the pancreas-a surgical disease. Nature reviews Gastroenterology & hepatology. 2012; **9**(5): 253-9.
72. Talamini MA, Pitt HA, Hruban RH, Boitnott JK, Coleman J, Cameron JL. Spectrum of cystic tumors of the pancreas. American journal of surgery. 1992; **163**(1): 117-23; discussion 23-4.
73. Takasu N, Kimura W, Moriya T, Hirai I, Takeshita A, Kamio Y, et al. Intraductal papillary-mucinous neoplasms of the gastric and intestinal types may have less malignant potential than the pancreatobiliary type. Pancreas. 2010; **39**(5): 604-10.
74. Shi C, Hruban RH. Intraductal papillary mucinous neoplasm. Human pathology. 2012; **43**(1): 1-16.
75. Fernandez-del Castillo C, Adsay NV. Intraductal papillary mucinous neoplasms of the pancreas. Gastroenterology. 2010; **139**(3): 708-13, 13 e1-2.
76. Mino-Kenudson M, Fernandez-del Castillo C, Baba Y, Valsangkar NP, Liss AS, Hsu M, et al. Prognosis of invasive intraductal papillary mucinous neoplasm depends on histological and precursor epithelial subtypes. Gut. 2011; **60**(12): 1712-20.
77. Adsay NV, Merati K, Basturk O, Iacobuzio-Donahue C, Levi E, Cheng JD, et al. Pathologically and biologically distinct types of epithelium in intraductal papillary mucinous neoplasms: delineation of an "intestinal" pathway of carcinogenesis in the pancreas. The American journal of surgical pathology. 2004; **28**(7): 839-48.
78. Luttges J, Zamboni G, Longnecker D, Kloppel G. The immunohistochemical mucin expression pattern distinguishes different types of intraductal papillary mucinous neoplasms of the pancreas and determines their relationship to mucinous noncystic carcinoma and ductal adenocarcinoma. The American journal of surgical pathology. 2001; **25**(7): 942-8.
79. Basturk O, Khayyata S, Klimstra DS, Hruban RH, Zamboni G, Coban I, et al. Preferential expression of MUC6 in oncocytic and pancreatobiliary types of intraductal papillary neoplasms highlights a pyloropancreatic pathway, distinct from the intestinal pathway, in pancreatic carcinogenesis. The American journal of surgical pathology. 2010; **34**(3): 364-70.
80. Yonezawa S, Taira M, Osako M, Kubo M, Tanaka S, Sakoda K, et al. MUC-1 mucin expression in invasive areas of intraductal papillary mucinous tumors of the pancreas. Pathology international. 1998; **48**(4): 319-22.
81. Terada T, Nakanuma Y. Expression of mucin carbohydrate antigens (T, Tn and sialyl Tn) and MUC-1 gene product in intraductal papillary-mucinous neoplasm of the pancreas. American journal of clinical pathology. 1996; **105**(5): 613-20.
82. Karasaki H, Mizukami Y, Tokusashi Y, Koizumi K, Ishizaki A, Imai K, et al. Localization of the most severely dysplastic/invasive lesions and mucin phenotypes in intraductal papillary mucinous neoplasm of the pancreas. Pancreas. 2011; **40**(4): 588-94.
83. Maker AV, Katabi N, Gonen M, DeMatteo RP, D'Angelica MI, Fong Y, et al. Pancreatic cyst fluid and serum mucin levels predict dysplasia in intraductal papillary mucinous neoplasms of the pancreas. Annals of surgical oncology. 2011; **18**(1): 199-206.
84. Klimstra DS. Cystic, mucin-producing neoplasms of the pancreas: the distinguishing features of mucinous cystic neoplasms and intraductal papillary mucinous neoplasms. Seminars in diagnostic pathology. 2005; **22**(4): 318-29.
85. Handra-Luca A, Flejou JF, Rufat P, Corcos O, Belghiti J, Ruszniewski P, et al. Human pancreatic mucinous cystadenoma is characterized by distinct mucin, cytokeratin and CD10 expression compared with intraductal papillary-mucinous adenoma. Histopathology. 2006; **48**(7): 813-21.
86. Satoh K, Shimosegawa T, Moriizumi S, Koizumi M, Toyota T. *KRAS* mutation and p53 protein accumulation in intraductal mucin-hypersecreting neoplasms of the pancreas. Pancreas. 1996; **12**(4): 362-8.

87. Wada K. p16 and p53 gene alterations and accumulations in the malignant evolution of intraductal papillary-mucinous tumors of the pancreas. Journal of hepato-biliary-pancreatic surgery. 2002; **9**(1): 76-85.
88. Kawahira H, Kobayashi S, Kaneko K, Asano T, Ochiai T. p53 protein expression in intraductal papillary mucinous tumors (IPMT) of the pancreas as an indicator of tumor malignancy. Hepato-gastroenterology. 2000; **47**(34): 973-7.
89. Amato E, Molin MD, Mafficini A, Yu J, Malleo G, Rusev B, et al. Targeted next-generation sequencing of cancer genes dissects the molecular profiles of intraductal papillary neoplasms of the pancreas. The Journal of pathology. 2014; **233**(3): 217-27.
90. Sasaki M, Sugio K, Kuwabara Y, Koga H, Nakagawa M, Chen T, et al. Alterations of tumor suppressor genes (Rb, p16, p27 and p53) and an increased FDG uptake in lung cancer. Annals of nuclear medicine. 2003; **17**(3): 189-96.
91. Biankin AV, Biankin SA, Kench JG, Morey AL, Lee CS, Head DR, et al. Aberrant p16(INK4A) and DPC4/Smad4 expression in intraductal papillary mucinous tumours of the pancreas is associated with invasive ductal adenocarcinoma. Gut. 2002; **50**(6): 861-8.
92. Fritz S, Buchler MW, Werner J. [Surgical therapy of intraductal papillary mucinous neoplasms of the pancreas]. Der Chirurg; Zeitschrift fur alle Gebiete der operativen Medizen. 2012; **83**(2): 130-5.
93. Sohn TA, Yeo CJ, Cameron JL, Hruban RH, Fukushima N, Campbell KA, et al. Intraductal papillary mucinous neoplasms of the pancreas: an updated experience. Annals of surgery. 2004; **239**(6): 788-97; discussion 97-9.
94. Wada K, Kozarek RA, Traverso LW. Outcomes following resection of invasive and noninvasive intraductal papillary mucinous neoplasms of the pancreas. American journal of surgery. 2005; **189**(5): 632-6; discussion 7.
95. Salvia R, Crippa S, Falconi M, Bassi C, Guarise A, Scarpa A, et al. Branch-duct intraductal papillary mucinous neoplasms of the pancreas: to operate or not to operate? Gut. 2007; **56**(8): 1086-90.
96. Abe K, Suda K, Arakawa A, Yamasaki S, Sonoue H, Mitani K, et al. Different patterns of p16INK4A and p53 protein expressions in intraductal papillary-mucinous neoplasms and pancreatic intraepithelial neoplasia. Pancreas. 2007; **34**(1): 85-91.
97. Conlon KC. Intraductal papillary mucinous tumors of the pancreas. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2005; **23**(20): 4518-23.
98. Fernandez-del Castillo C, Targarona J, Thayer SP, Rattner DW, Brugge WR, Warshaw AL. Incidental pancreatic cysts: clinicopathologic characteristics and comparison with symptomatic patients. Arch Surg. 2003; **138**(4): 427-3; discussion 33-4.
99. Sarr MG, Murr M, Smyrk TC, Yeo CJ, Fernandez-del-Castillo C, Hawes RH, et al. Primary cystic neoplasms of the pancreas. Neoplastic disorders of emerging importance-current state-of-the-art and unanswered questions. Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract. 2003; **7**(3): 417-28.
100. Khan S, Sclabas G, Reid-Lombardo KM. Population-based epidemiology, risk factors and screening of intraductal papillary mucinous neoplasm patients. World journal of gastrointestinal surgery. 2010; **2**(10): 314-8.
101. Lee CJ, Scheiman J, Anderson MA, Hines OJ, Reber HA, Farrell J, et al. Risk of malignancy in resected cystic tumors of the pancreas < or =3 cm in size: is it safe to observe asymptomatic patients? A multi-institutional report. Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract. 2008; **12**(2): 234-42.
102. Yoon WJ, Ryu JK, Lee JK, Woo SM, Lee SH, Park JK, et al. Extrapancreatic malignancies in patients with intraductal papillary mucinous neoplasm of the pancreas: prevalence, associated factors, and comparison with patients with other pancreatic cystic neoplasms. Annals of surgical oncology. 2008; **15**(11): 3193-8.

103. Woo SM, Ryu JK, Lee SH, Yoon WJ, Kim YT, Yoon YB. Branch duct intraductal papillary mucinous neoplasms in a retrospective series of 190 patients. The British journal of surgery. 2009; **96**(4): 405-11.
104. Tanaka M, Fernandez-Del Castillo C, Adsay V, Chari S, Falconi M, Jang JY, et al. International consensus guidelines 2012 for the management of IPMN and MCN of the pancreas. Pancreatology. 2012; **12**(3): 183-97.
105. Whitcomb DC, Gorry MC, Preston RA, Furey W, Sossenheimer MJ, Ulrich CD, et al. Hereditary pancreatitis is caused by a mutation in the cationic trypsinogen gene. Nature genetics. 1996; **14**(2): 141-5.
106. Witt H, Luck W, Hennies HC, Classen M, Kage A, Lass U, et al. Mutations in the gene encoding the serine protease inhibitor, Kazal type 1 are associated with chronic pancreatitis. Nature genetics. 2000; **25**(2): 213-6.
107. Ravnik-Glavac M, Glavac D, di Sant' Agnese P, Chernick M, Dean M. Cystic fibrosis gene mutations detected in hereditary pancreatitis. Pflugers Archiv : European journal of physiology. 1996; **431**(6 Suppl 2): R191-2.
108. Grocock CJ, Rebours V, Delhaye MN, Andren-Sandberg A, Weiss FU, Mountford R, et al. The variable phenotype of the p.A16V mutation of cationic trypsinogen (PRSS1) in pancreatitis families. Gut. 2010; **59**(3): 357-63.
109. Lynch HT, Fitzsimmons ML, Smyrk TC, Lanspa SJ, Watson P, McClellan J, et al. Familial pancreatic cancer: clinicopathologic study of 18 nuclear families. The American journal of gastroenterology. 1990; **85**(1): 54-60.
110. MacDermott RP, Kramer P. Adenocarcinoma of the pancreas in four siblings. Gastroenterology. 1973; **65**(1): 137-9.
111. Reimer RR, Fraumeni JF, Jr., Ozols RF, Bender R. Pancreatic cancer in father and son. Lancet. 1977; **1**(8017): 911.
112. Ehrenthal D, Haeger L, Griffin T, Compton C. Familial pancreatic adenocarcinoma in three generations. A case report and a review of the literature. Cancer. 1987; **59**(9): 1661-4.
113. Rulyak SJ, Brentnall TA. Inherited pancreatic cancer: improvements in our understanding of genetics and screening. The international journal of biochemistry & cell biology. 2004; **36**(8): 1386-92.
114. Canto MI, Hruban RH, Fishman EK, Kamel IR, Schulick R, Zhang Z, et al. Frequent detection of pancreatic lesions in asymptomatic high-risk individuals. Gastroenterology. 2012; **142**(4): 796-804; quiz e14-5.
115. Canto MI, Harinck F, Hruban RH, Offerhaus GJ, Poley JW, Kamel I, et al. International Cancer of the Pancreas Screening (CAPS) Consortium summit on the management of patients with increased risk for familial pancreatic cancer. Gut. 2013; **62**(3): 339-47.
116. Langer P, Kann PH, Fendrich V, Habbe N, Schneider M, Sina M, et al. Five years of prospective screening of high-risk individuals from families with familial pancreatic cancer. Gut. 2009; **58**(10): 1410-8.
117. Poley JW, Kluijt I, Gouma DJ, Harinck F, Wagner A, Aalfs C, et al. The yield of first-time endoscopic ultrasonography in screening individuals at a high risk of developing pancreatic cancer. The American journal of gastroenterology. 2009; **104**(9): 2175-81.
118. Howes N, Lerch MM, Greenhalf W, Stocken DD, Ellis I, Simon P, et al. Clinical and genetic characteristics of hereditary pancreatitis in Europe. Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association. 2004; **2**(3): 252-61.
119. Whitcomb DC. Hereditary pancreatitis: a model for understanding the genetic basis of acute and chronic pancreatitis. Pancreatology. 2001; **1**(6): 565-70.
120. Vitone LJ, Greenhalf W, Howes NR, Neoptolemos JP. Hereditary pancreatitis and secondary screening for early pancreatic cancer. Rocz Akad Med Bialymst. 2005; **50**: 73-84.
121. de las Heras-Castano G, Castro-Senosiain B, Fontalba A, Lopez-Hoyos M, Sanchez-Juan P. Hereditary pancreatitis: clinical features and inheritance characteristics of the R122C mutation in the

cationic trypsinogen gene (PRSS1) in six Spanish families. JOP : Journal of the pancreas. 2009; **10**(3): 249-55.
122. Sossenheimer MJ, Aston CE, Preston RA, Gates LK, Jr., Ulrich CD, Martin SP, et al. Clinical characteristics of hereditary pancreatitis in a large family, based on high-risk haplotype. The Midwest Multicenter Pancreatic Study Group (MMPSG). The American journal of gastroenterology. 1997; **92**(7): 1113-6.
123. Rebours V, Boutron-Ruault MC, Schnee M, Ferec C, Le Marechal C, Hentic O, et al. The natural history of hereditary pancreatitis: a national series. Gut. 2009; **58**(1): 97-103.
124. Howes N, Lerch MM, Greenhalf W, Stocken DD, Ellis I, Simon P, et al. Clinical and genetic characteristics of hereditary pancreatitis in Europe. Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association. 2004; **2**(3): 252-61.
125. Cohn JA, Friedman KJ, Noone PG, Knowles MR, Silverman LM, Jowell PS. Relation between mutations of the cystic fibrosis gene and idiopathic pancreatitis. The New England journal of medicine. 1998; **339**(10): 653-8.
126. Brand RE, Lerch MM, Rubinstein WS, Neoptolemos JP, Whitcomb DC, Hruban RH, et al. Advances in counselling and surveillance of patients at risk for pancreatic cancer. Gut. 2007; **56**(10): 1460-9.
127. Klein AP, Beaty TH, Bailey-Wilson JE, Brune KA, Hruban RH, Petersen GM. Evidence for a major gene influencing risk of pancreatic cancer. Genetic epidemiology. 2002; **23**(2): 133-49.
128. Rulyak SJ, Lowenfels AB, Maisonneuve P, Brentnall TA. Risk factors for the development of pancreatic cancer in familial pancreatic cancer kindreds. Gastroenterology. 2003; **124**(5): 1292-9.
129. McFaul CD, Greenhalf W, Earl J, Howes N, Neoptolemos JP, Kress R, et al. Anticipation in familial pancreatic cancer. Gut. 2006; **55**(2): 252-8.
130. Schneider R, Slater EP, Sina M, Habbe N, Fendrich V, Matthai E, et al. German national case collection for familial pancreatic cancer (FaPaCa): ten years experience. Familial cancer. 2011; **10**(2): 323-30.
131. Eberle MA, Pfutzer R, Pogue-Geile KL, Bronner MP, Crispin D, Kimmey MB, et al. A new susceptibility locus for autosomal dominant pancreatic cancer maps to chromosome 4q32-34. American journal of human genetics. 2002; **70**(4): 1044-8.
132. Pogue-Geile KL, Chen R, Bronner MP, Crnogorac-Jurcevic T, Moyes KW, Dowen S, et al. Palladin mutation causes familial pancreatic cancer and suggests a new cancer mechanism. PLoS medicine. 2006; **3**(12): e516.
133. Klein AP, Brune KA, Petersen GM, Goggins M, Tersmette AC, Offerhaus GJ, et al. Prospective risk of pancreatic cancer in familial pancreatic cancer kindreds. Cancer research. 2004; **64**(7): 2634-8.
134. Bartsch DK, Gress TM, Langer P. Familial pancreatic cancer-current knowledge. Nature reviews Gastroenterology & hepatology. 2012; **9**(8): 445-53.
135. Lynch HT, Brand RE, Deters CA, Fusaro RM. Update on familial pancreatic cancer. Current gastroenterology reports. 2001; **3**(2): 121-8.
136. Hruban RH, Petersen GM, Goggins M, Tersmette AC, Offerhaus GJ, Falatko F, et al. Familial pancreatic cancer. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 1999; **10 Suppl 4**: 69-73.
137. Yachida S, Jones S, Bozic I, Antal T, Leary R, Fu B, et al. Distant metastasis occurs late during the genetic evolution of pancreatic cancer. Nature. 2010; **467**(7319): 1114-7.
138. Klein AP. Identifying people at a high risk of developing pancreatic cancer. Nature reviews Cancer. 2013; **13**(1): 66-74.
139. Yan L, McFaul C, Howes N, Leslie J, Lancaster G, Wong T, et al. Molecular analysis to detect pancreatic ductal adenocarcinoma in high-risk groups. Gastroenterology. 2005; **128**(7): 2124-30.
140. Greenhalf W, Grocock C, Harcus M, Neoptolemos J. Screening of high-risk families for pancreatic cancer. Pancreatology. 2009; **9**(3): 215-22.
141. Tuveson DA, Neoptolemos JP. Understanding metastasis in pancreatic cancer: a call for new clinical approaches. Cell. 2012; **148**(1-2): 21-3.

142. Hunt GC, Faigel DO. Assessment of EUS for diagnosing, staging, and determining resectability of pancreatic cancer: a review. Gastrointestinal endoscopy. 2002; **55**(2): 232-7.
143. Bronstein YL, Loyer EM, Kaur H, Choi H, David C, DuBrow RA, et al. Detection of small pancreatic tumors with multiphasic helical CT. AJR American journal of roentgenology. 2004; **182**(3): 619-23.
144. Win AK, Young JP, Lindor NM, Tucker KM, Ahnen DJ, Young GP, et al. Colorectal and other cancer risks for carriers and noncarriers from families with a DNA mismatch repair gene mutation: a prospective cohort study. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2012; **30**(9): 958-64.
145. Lindor NM, Petersen GM, Spurdle AB, Thompson B, Goldgar DE, Thibodeau SN. Pancreatic cancer and a novel MSH2 germline alteration. Pancreas. 2011; **40**(7): 1138-40.
146. Hofstatter EW, Domchek SM, Miron A, Garber J, Wang M, Componeschi K, et al. PALB2 mutations in familial breast and pancreatic cancer. Familial cancer. 2011; **10**(2): 225-31.
147. Harinck F, Canto MI, Schulick R, Goggins M, Poley JW, Fockens P, et al. Surveillance in individuals at high risk of pancreatic cancer: too early to tell? Gut. 2010; **59**(7): 1005; author reply 6-7.
148. Nair RM, Barthel JS, Centeno BA, Choi J, Klapman JB, Malafa MP. Interdisciplinary management of an intraductal papillary mucinous neoplasm of the pancreas. Cancer control : journal of the Moffitt Cancer Center. 2008; **15**(4): 322-33.
149. Ahmad NA, Lewis JD, Siegelman ES, Rosato EF, Ginsberg GG, Kochman ML. Role of endoscopic ultrasound and magnetic resonance imaging in the preoperative staging of pancreatic adenocarcinoma. The American journal of gastroenterology. 2000; **95**(8): 1926-31.
150. Canto MI, Goggins M, Hruban RH, Petersen GM, Giardiello FM, Yeo C, et al. Screening for early pancreatic neoplasia in high-risk individuals: a prospective controlled study. Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association. 2006; **4**(6): 766-81; quiz 665.
151. Kim JH, Hong SS, Kim YJ, Kim JK, Eun HW. Intraductal papillary mucinous neoplasm of the pancreas: differentiate from chronic pancreatits by MR imaging. European journal of radiology. 2012; **81**(4): 671-6.
152. Singh S, Tang SJ, Sreenarasimhaiah J, Lara LF, Siddiqui A. The clinical utility and limitations of serum carbohydrate antigen (CA19-9) as a diagnostic tool for pancreatic cancer and cholangiocarcinoma. Digestive diseases and sciences. 2011; **56**(8): 2491-6.
153. Grocock CJ, Vitone LJ, Harcus MJ, Neoptolemos JP, Raraty MG, Greenhalf W. Familial pancreatic cancer: a review and latest advances. Advances in medical sciences. 2007; **52**: 37-49.
154. Lane DP. Cell immortalization and transformation by the p53 gene. Nature. 1984; **312**(5995): 596-7.
155. Vousden KH, Lane DP. p53 in health and disease. Nature reviews Molecular cell biology. 2007; **8**(4): 275-83.
156. Eshuis WJ, van der Gaag NA, Rauws EA, van Eijck CH, Bruno MJ, Kuipers EJ, et al. Therapeutic delay and survival after surgery for cancer of the pancreatic head with or without preoperative biliary drainage. Annals of surgery. 2010; **252**(5): 840-9.
157. el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, et al. WAF1, a potential mediator of p53 tumor suppression. Cell. 1993; **75**(4): 817-25.
158. Funk WD, Pak DT, Karas RH, Wright WE, Shay JW. A transcriptionally active DNA-binding site for human p53 protein complexes. Molecular and cellular biology. 1992; **12**(6): 2866-71.
159. Bourdon JC, Deguin-Chambon V, Lelong JC, Dessen P, May P, Debuire B, et al. Further characterisation of the p53 responsive element--identification of new candidate genes for trans-activation by p53. Oncogene. 1997; **14**(1): 85-94.
160. Bourdon JC, Renzing J, Robertson PL, Fernandes KN, Lane DP. Scotin, a novel p53-inducible proapoptotic protein located in the ER and the nuclear membrane. The Journal of cell biology. 2002; **158**(2): 235-46.

161. Okorokov AL, Sherman MB, Plisson C, Grinkevich V, Sigmundsson K, Selivanova G, et al. The structure of p53 tumour suppressor protein reveals the basis for its functional plasticity. The EMBO journal. 2006; **25**(21): 5191-200.
162. Green DR, Kroemer G. Cytoplasmic functions of the tumour suppressor p53. Nature. 2009; **458**(7242): 1127-30.
163. Vaseva AV, Moll UM. The mitochondrial p53 pathway. Biochimica et biophysica acta. 2009; **1787**(5): 414-20.
164. Olivier M, Hollstein M, Hainaut P. *TP53* mutations in human cancers: origins, consequences, and clinical use. Cold Spring Harbor perspectives in biology. 2010; **2**(1): a001008.
165. Armstrong JF, Kaufman MH, Harrison DJ, Clarke AR. High-frequency developmental abnormalities in p53-deficient mice. Current biology : CB. 1995; **5**(8): 931-6.
166. Sah VP, Attardi LD, Mulligan GJ, Williams BO, Bronson RT, Jacks T. A subset of p53-deficient embryos exhibit exencephaly. Nature genetics. 1995; **10**(2): 175-80.
167. Hu W, Feng Z, Teresky AK, Levine AJ. p53 regulates maternal reproduction through LIF. Nature. 2007; **450**(7170): 721-4.
168. Cui R, Widlund HR, Feige E, Lin JY, Wilensky DL, Igras VE, et al. Central role of p53 in the suntan response and pathologic hyperpigmentation. Cell. 2007; **128**(5): 853-64.
169. McGowan KA, Li JZ, Park CY, Beaudry V, Tabor HK, Sabnis AJ, et al. Ribosomal mutations cause p53-mediated dark skin and pleiotropic effects. Nature genetics. 2008; **40**(8): 963-70.
170. Matheu A, Maraver A, Serrano M. The Arf/p53 pathway in cancer and aging. Cancer research. 2008; **68**(15): 6031-4.
171. Matheu A, Maraver A, Klatt P, Flores I, Garcia-Cao I, Borras C, et al. Delayed ageing through damage protection by the Arf/p53 pathway. Nature. 2007; **448**(7151): 375-9.
172. Minamino T, Orimo M, Shimizu I, Kunieda T, Yokoyama M, Ito T, et al. A crucial role for adipose tissue p53 in the regulation of insulin resistance. Nature medicine. 2009; **15**(9): 1082-7.
173. Liu P, Xu B, Cavalieri TA, Hock CE. Pifithrin-alpha attenuates p53-mediated apoptosis and improves cardiac function in response to myocardial ischemia/reperfusion in aged rats. Shock. 2006; **26**(6): 608-14.
174. Morrison RS, Kinoshita Y, Johnson MD, Guo W, Garden GA. p53-dependent cell death signaling in neurons. Neurochemical research. 2003; **28**(1): 15-27.
175. Bae BI, Xu H, Igarashi S, Fujimuro M, Agrawal N, Taya Y, et al. p53 mediates cellular dysfunction and behavioral abnormalities in Huntington's disease. Neuron. 2005; **47**(1): 29-41.
176. Bretaud S, Allen C, Ingham PW, Bandmann O. p53-dependent neuronal cell death in a DJ-1-deficient zebrafish model of Parkinson's disease. Journal of neurochemistry. 2007; **100**(6): 1626-35.
177. Culmsee C, Mattson MP. p53 in neuronal apoptosis. Biochemical and biophysical research communications. 2005; **331**(3): 761-77.
178. Duan W, Ding H, Subler MA, Zhu WG, Zhang H, Stoner GD, et al. Lung-specific expression of human mutant p53-273H is associated with a high frequency of lung adenocarcinoma in transgenic mice. Oncogene. 2002; **21**(51): 7831-8.
179. Oren M, Rotter V. Mutant p53 gain-of-function in cancer. Cold Spring Harbor perspectives in biology. 2010; **2**(2): a001107.
180. Bourdon JC, Laurenzi VD, Melino G, Lane D. p53: 25 years of research and more questions to answer. Cell death and differentiation. 2003; **10**(4): 397-9.
181. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. Science. 1991; **253**(5015): 49-53.
182. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature. 2000; **408**(6810): 307-10.
183. Irwin MS, Kaelin WG. p53 family update: p73 and p63 develop their own identities. Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research. 2001; **12**(7): 337-49.
184. Smith ML, Fornace AJ, Jr. Genomic instability and the role of p53 mutations in cancer cells. Current opinion in oncology. 1995; **7**(1): 69-75.

185. Chang F, Syrjanen S, Syrjanen K. Implications of the p53 tumor-suppressor gene in clinical oncology. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 1995; **13**(4): 1009-22.
186. Mraz M, Malinova K, Kotaskova J, Pavlova S, Tichy B, Malcikova J, et al. miR-34a, miR-29c and miR-17-5p are downregulated in CLL patients with *TP53* abnormalities. Leukemia. 2009; **23**(6): 1159-63.
187. Dolezalova D, Mraz M, Barta T, Plevova K, Vinarsky V, Holubcova Z, et al. MicroRNAs regulate p21(Waf1/Cip1) protein expression and the DNA damage response in human embryonic stem cells. Stem Cells. 2012; **30**(7): 1362-72.
188. Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell. 1993; **75**(4): 805-16.
189. Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. p21 is a universal inhibitor of cyclin kinases. Nature. 1993; **366**(6456): 701-4.
190. Lane DP. Cancer. p53, guardian of the genome. Nature. 1992; **358**(6381): 15-6.
191. Ortiz-Hidalgo C, De La Vega G, Aguirre-Garcia J. The histopathology and biologic prognostic factors of Barrett's esophagus: a review. Journal of clinical gastroenterology. 1998; **26**(4): 324-33.
192. Harris CC, Hollstein M. Clinical implications of the p53 tumor-suppressor gene. The New England journal of medicine. 1993; **329**(18): 1318-27.
193. Vousden KH, Lu X. Live or let die: the cell's response to p53. Nature reviews Cancer. 2002; **2**(8): 594-604.
194. Vousden KH. Activation of the p53 tumor suppressor protein. Biochimica et biophysica acta. 2002; **1602**(1): 47-59.
195. Levine AJ. p53, the cellular gatekeeper for growth and division. Cell. 1997; **88**(3): 323-31.
196. Ambs S, Hussain SP, Marrogi AJ, Harris CC. Cancer-prone oxyradical overload disease. IARC scientific publications. 1999; (150): 295-302.
197. Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW, et al. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature. 1996; **379**(6560): 88-91.
198. Brady CA, Attardi LD. p53 at a glance. Journal of cell science. 2010; **123**(Pt 15): 2527-32.
199. Tasdemir E, Chiara Maiuri M, Morselli E, Criollo A, D'Amelio M, Djavaheri-Mergny M, et al. A dual role of p53 in the control of autophagy. Autophagy. 2008; **4**(6): 810-4.
200. Tasdemir E, Maiuri MC, Galluzzi L, Vitale I, Djavaheri-Mergny M, D'Amelio M, et al. Regulation of autophagy by cytoplasmic p53. Nature cell biology. 2008; **10**(6): 676-87.
201. Kastan MB, Bartek J. Cell-cycle checkpoints and cancer. Nature. 2004; **432**(7015): 316-23.
202. Gorgoulis VG, Vassiliou LV, Karakaidos P, Zacharatos P, Kotsinas A, Liloglou T, et al. Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. Nature. 2005; **434**(7035): 907-13.
203. Bartkova J, Horejsi Z, Koed K, Kramer A, Tort F, Zieger K, et al. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature. 2005; **434**(7035): 864-70.
204. Kenzelmann Broz D, Attardi LD. In vivo analysis of p53 tumor suppressor function using genetically engineered mouse models. Carcinogenesis. 2010; **31**(8): 1311-8.
205. Soussi T, Beroud C. Assessing *TP53* status in human tumours to evaluate clinical outcome. Nature reviews Cancer. 2001; **1**(3): 233-40.
206. Sherr CJ. Tumor surveillance via the ARF-p53 pathway. Genes & development. 1998; **12**(19): 2984-91.
207. Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA, Jr., Butel JS, et al. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature. 1992; **356**(6366): 215-21.
208. Vousden KH, Ryan KM. p53 and metabolism. Nature reviews Cancer. 2009; **9**(10): 691-700.

209. Weissmueller S, Manchado E, Saborowski M, Morris JPt, Wagenblast E, Davis CA, et al. Mutant p53 drives pancreatic cancer metastasis through cell-autonomous PDGF receptor beta signaling. Cell. 2014; **157**(2): 382-94.
210. Greenblatt MS, Bennett WP, Hollstein M, Harris CC. Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer research. 1994; **54**(18): 4855-78.
211. Kim TG, Chen J, Sadoshima J, Lee Y. Jumonji represses atrial natriuretic factor gene expression by inhibiting transcriptional activities of cardiac transcription factors. Molecular and cellular biology. 2004; **24**(23): 10151-60.
212. Kim E, Deppert W. Transcriptional activities of mutant p53: when mutations are more than a loss. Journal of cellular biochemistry. 2004; **93**(5): 878-86.
213. Pan WH, Chen JW, Fann C, Jou YS, Wu SY. Linkage analysis with candidate genes: the Taiwan young-onset hypertension genetic study. Human genetics. 2000; **107**(3): 210-5.
214. Friedlander P, Haupt Y, Prives C, Oren M. A mutant p53 that discriminates between p53-responsive genes cannot induce apoptosis. Molecular and cellular biology. 1996; **16**(9): 4961-71.
215. Ludwig RL, Bates S, Vousden KH. Differential activation of target cellular promoters by p53 mutants with impaired apoptotic function. Molecular and cellular biology. 1996; **16**(9): 4952-60.
216. Masciarelli S, Fontemaggi G, Di Agostino S, Donzelli S, Carcarino E, Strano S, et al. Gain-of-function mutant p53 downregulates miR-223 contributing to chemoresistance of cultured tumor cells. Oncogene. 2014; **33**(12): 1601-8.
217. Gudkov AV, Komarova EA. The role of p53 in determining sensitivity to radiotherapy. Nature reviews Cancer. 2003; **3**(2): 117-29.
218. Ho J, Benchimol S. Transcriptional repression mediated by the p53 tumour suppressor. Cell death and differentiation. 2003; **10**(4): 404-8.
219. Guttman M, Amit I, Garber M, French C, Lin MF, Feldser D, et al. Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. Nature. 2009; **458**(7235): 223-7.
220. Caelles C, Helmberg A, Karin M. p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes. Nature. 1994; **370**(6486): 220-3.
221. Joerger AC, Fersht AR. Structural biology of the tumor suppressor p53 and cancer-associated mutants. Advances in cancer research. 2007; **97**: 1-23.
222. Joerger AC, Fersht AR. Structure-function-rescue: the diverse nature of common p53 cancer mutants. Oncogene. 2007; **26**(15): 2226-42.
223. Soussi T, Lozano G. p53 mutation heterogeneity in cancer. Biochemical and biophysical research communications. 2005; **331**(3): 834-42.
224. Slee EA, O'Connor DJ, Lu X. To die or not to die: how does p53 decide? Oncogene. 2004; **23**(16): 2809-18.
225. Li HH, Li AG, Sheppard HM, Liu X. Phosphorylation on Thr-55 by TAF1 mediates degradation of p53: a role for TAF1 in cell G1 progression. Molecular cell. 2004; **13**(6): 867-78.
226. Hidalgo M. Pancreatic cancer. The New England journal of medicine. 2010; **362**(17): 1605-17.
227. Boschman CR, Stryker S, Reddy JK, Rao MS. Expression of p53 protein in precursor lesions and adenocarcinoma of human pancreas. The American journal of pathology. 1994; **145**(6): 1291-5.
228. Rhim AD, Stanger BZ. Molecular biology of pancreatic ductal adenocarcinoma progression: aberrant activation of developmental pathways. Progress in molecular biology and translational science. 2010; **97**: 41-78.
229. Maitra A, Adsay NV, Argani P, Iacobuzio-Donahue C, De Marzo A, Cameron JL, et al. Multicomponent analysis of the pancreatic adenocarcinoma progression model using a pancreatic intraepithelial neoplasia tissue microarray. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc. 2003; **16**(9): 902-12.
230. Morton JP, Jamieson NB, Karim SA, Athineos D, Ridgway RA, Nixon C, et al. LKB1 haploinsufficiency cooperates with Kras to promote pancreatic cancer through suppression of p21-dependent growth arrest. Gastroenterology. 2010; **139**(2): 586-97, 97 e1-6.

231. Weissmuller T, Glover LE, Fennimore B, Curtis VF, MacManus CF, Ehrentraut SF, et al. HIF-dependent regulation of AKAP12 (gravin) in the control of human vascular endothelial function. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2014; **28**(1): 256-64.
232. Sato Y, Mukai M, Sasaki M, Kitao A, Yoneda N, Kobayashi D, et al. Intraductal papillary-mucinous neoplasm of the pancreas associated with polycystic liver and kidney disease. Pathology international. 2009; **59**(3): 201-4.
233. Ihrler S, Weiler C, Hirschmann A, Sendelhofert A, Lang S, Guntinas-Lichius O, et al. Intraductal carcinoma is the precursor of carcinoma ex pleomorphic adenoma and is often associated with dysfunctional p53. Histopathology. 2007; **51**(3): 362-71.
234. Adsay NV. Intraductal papillary mucinous neoplasms of the pancreas: pathology and molecular genetics. Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract. 2002; **6**(5): 656-9.
235. Li A, Yu J, Kim H, Wolfgang CL, Canto MI, Hruban RH, et al. MicroRNA array analysis finds elevated serum miR-1290 accurately distinguishes patients with low-stage pancreatic cancer from healthy and disease controls. Clinical cancer research : an official journal of the American Association for Cancer Research. 2013; **19**(13): 3600-10.
236. Kanda M, Knight S, Topazian MD, Syngal S, Farrell J, Lee JH, et al. Mutant GNAS detected in duodenal collections of secretin-stimulated pancreatic juice indicates the presence or emergence of pancreatic cysts. Gut. 2012.
237. Fritz S, Klauss M, Bergmann F, Hackert T, Hartwig W, Strobel O, et al. Small (Sendai Negative) Branch-Duct IPMNs: Not Harmless. Annals of surgery. 2012; **256**(2): 313-20.
238. Bassi C, Stocken DD, Olah A, Friess H, Buckels J, Hickey H, et al. Influence of surgical resection and post-operative complications on survival following adjuvant treatment for pancreatic cancer in the ESPAC-1 randomized controlled trial. Digestive surgery. 2005; **22**(5): 353-63.
239. Carter R, Stocken DD, Ghaneh P, Bramhall SR, Olah A, Kelemen D, et al. Longitudinal quality of life data can provide insights on the impact of adjuvant treatment for pancreatic cancer-Subset analysis of the ESPAC-1 data. International journal of cancer Journal international du cancer. 2009; **124**(12): 2960-5.
240. Greenhalf W, Ghaneh P, Neoptolemos JP, Palmer DH, Cox TF, Lamb RF, et al. Pancreatic cancer hENT1 expression and survival from gemcitabine in patients from the ESPAC-3 trial. Journal of the National Cancer Institute. 2014; **106**(1): djt347.
241. Neoptolemos JP, Kerr DJ, Beger H, Link K, Pederzoli P, Bassi C, et al. ESPAC-1 trial progress report: the European randomized adjuvant study comparing radiochemotherapy, 6 months chemotherapy and combination therapy versus observation in pancreatic cancer. Digestion. 1997; **58**(6): 570-7.
242. Neoptolemos JP, Moore MJ, Cox TF, Valle JW, Palmer DH, McDonald AC, et al. Effect of adjuvant chemotherapy with fluorouracil plus folinic acid or gemcitabine vs observation on survival in patients with resected periampullary adenocarcinoma: the ESPAC-3 periampullary cancer randomized trial. JAMA : the journal of the American Medical Association. 2012; **308**(2): 147-56.
243. Neoptolemos JP, Stocken DD, Bassi C, Ghaneh P, Cunningham D, Goldstein D, et al. Adjuvant chemotherapy with fluorouracil plus folinic acid vs gemcitabine following pancreatic cancer resection: a randomized controlled trial. JAMA : the journal of the American Medical Association. 2010; **304**(10): 1073-81.
244. Manchester KL. Value of A260/A280 ratios for measurement of purity of nucleic acids. BioTechniques. 1995; **19**(2): 208-10.
245. Huberman JA. Importance of measuring nucleic acid absorbance at 240 nm as well as at 260 and 280 nm. BioTechniques. 1995; **18**(4): 636.
246. Glasel JA. Validity of nucleic acid purities monitored by 260nm/280nm absorbance ratios. BioTechniques. 1995; **18**(1): 62-3.

247. Manchester KL. Use of UV methods for measurement of protein and nucleic acid concentrations. BioTechniques. 1996; **20**(6): 968-70.
248. DeAngelis MM, Wang DG, Hawkins TL. Solid-phase reversible immobilization for the isolation of PCR products. Nucleic acids research. 1995; **23**(22): 4742-3.
249. Nicholson JA, Greenhalf W, Jackson R, Cox TF, Butler JV, Hanna T, et al. Incidence of Post-ERCP Pancreatitis From Direct Pancreatic Juice Collection in Hereditary Pancreatitis and Familial Pancreatic Cancer Before and After the Introduction of Prophylactic Pancreatic Stents and Rectal Diclofenac. Pancreas. 2015; **44**(2): 260-5.
250. Whitcomb DC, Larusch J, Krasinskas AM, Klei L, Smith JP, Brand RE, et al. Common genetic variants in the CLDN2 and PRSS1-PRSS2 loci alter risk for alcohol-related and sporadic pancreatitis. Nature genetics. 2012.
251. Bradley EL, 3rd. A clinically based classification system for acute pancreatitis. Summary of the International Symposium on Acute Pancreatitis, Atlanta, Ga, September 11 through 13, 1992. Arch Surg. 1993; **128**(5): 586-90.
252. McFaul CD, Greenhalf W, Earl J, Howes N, Neoptolemos JP, Kress R, et al. Anticipation in familial pancreatic cancer. Gut. 2006; **55**(2): 252-8.
253. Threadgold J, Greenhalf W, Ellis I, Howes N, Lerch MM, Simon P, et al. The N34S mutation of SPINK1 (PSTI) is associated with a familial pattern of idiopathic chronic pancreatitis but does not cause the disease. Gut. 2002; **50**(5): 675-81.
254. Elmunzer BJ, Waljee AK, Elta GH, Taylor JR, Fehmi SM, Higgins PD. A meta-analysis of rectal NSAIDs in the prevention of post-ERCP pancreatitis. Gut. 2008; **57**(9): 1262-7.
255. Singh P, Das A, Isenberg G, Wong RC, Sivak MV, Jr., Agrawal D, et al. Does prophylactic pancreatic stent placement reduce the risk of post-ERCP acute pancreatitis? A meta-analysis of controlled trials. Gastrointestinal endoscopy. 2004; **60**(4): 544-50.
256. Raty S, Sand J, Pulkkinen M, Matikainen M, Nordback I. Post-ERCP pancreatitis: reduction by routine antibiotics. Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract. 2001; **5**(4): 339-45; discussion 45.
257. Singh P, Gurudu SR, Davidoff S, Sivak MV, Jr., Indaram A, Kasmin FE, et al. Sphincter of Oddi manometry does not predispose to post-ERCP acute pancreatitis. Gastrointestinal endoscopy. 2004; **59**(4): 499-505.
258. Choudari CP, Nickl NJ, Fogel E, Lehman GA, Sherman S. Hereditary pancreatitis: clinical presentation, ERCP findings, and outcome of endoscopic therapy. Gastrointestinal endoscopy. 2002; **56**(1): 66-71.
259. Dever JB, Irani S, Brandabur J, Traverso LW, Kozarek R. Outcomes of interventional ERCP in hereditary pancreatitis. Journal of clinical gastroenterology. 2010; **44**(1): 46-51.
260. Freeman ML. Role of pancreatic stents in prevention of post-ERCP pancreatitis. JOP : Journal of the pancreas. 2004; **5**(5): 322-7.
261. Elmunzer BJ, Scheiman JM, Lehman GA, Chak A, Mosler P, Higgins PD, et al. A randomized trial of rectal indomethacin to prevent post-ERCP pancreatitis. The New England journal of medicine. 2012; **366**(15): 1414-22.
262. Ito K, Fujita N, Kanno A, Matsubayashi H, Okaniwa S, Nakahara K, et al. Risk factors for post-ERCP pancreatitis in high risk patients who have undergone prophylactic pancreatic duct stenting: a multicenter retrospective study. Intern Med. 2011; **50**(24): 2927-32.
263. Nicholson JA, Greenhalf W, Jackson R, Cox TF, Butler JV, Hanna T, et al. Incidence of Post-ERCP Pancreatitis From Direct Pancreatic Juice Collection in Hereditary Pancreatitis and Familial Pancreatic Cancer Before and After the Introduction of Prophylactic Pancreatic Stents and Rectal Diclofenac. Pancreas. 9000; **Publish Ahead of Print**: 10.1097/MPA.0000000000000246.
264. Zhou W, Li Y, Zhang Q, Li X, Meng W, Zhang L, et al. Risk factors for postendoscopic retrograde cholangiopancreatography pancreatitis: a retrospective analysis of 7,168 cases. Pancreatology. 2011; **11**(4): 399-405.

265. Jowell PS, Branch MS, Fein SH, Purich ED, Kilaru R, Robuck G, et al. Intravenous synthetic secretin reduces the incidence of pancreatitis induced by endoscopic retrograde cholangiopancreatography. Pancreas. 2011; **40**(4): 533-9.
266. Ohhashi T, Azuma T, Roddie IC. Effect of potassium on adrenergic nerve endings in bovine mesenteric lymphatics. Journal of the autonomic nervous system. 1983; **8**(3): 205-11.
267. Choudhary A, Bechtold ML, Arif M, Szary NM, Puli SR, Othman MO, et al. Pancreatic stents for prophylaxis against post-ERCP pancreatitis: a meta-analysis and systematic review. Gastrointestinal endoscopy. 2011; **73**(2): 275-82.
268. Elmunzer BJ, Scheiman JM, Lehman GA, Chak A, Mosler P, Higgins PD, et al. A randomized trial of rectal indomethacin to prevent post-ERCP pancreatitis. The New England journal of medicine. 2012; **366**(15): 1414-22.
269. Sethi S, Sethi N, Wadhwa V, Garud S, Brown A. A meta-analysis on the role of rectal diclofenac and indomethacin in the prevention of post-endoscopic retrograde cholangiopancreatography pancreatitis. Pancreas. 2014; **43**(2): 190-7.
270. Furuya N, Kawa S, Akamatsu T, Furihata K. Long-term follow-up of patients with chronic pancreatitis and *KRAS* gene mutation detected in pancreatic juice. Gastroenterology. 1997; **113**(2): 593-8.
271. Bartsch DK, Gress TM, Langer P. Familial pancreatic cancer--current knowledge. Nature reviews Gastroenterology & hepatology. 2012; **9**(8): 445-53.
272. Kamisawa T, Tu Y, Egawa N, Nakajima H, Tsuruta K, Okamoto A. Malignancies associated with intraductal papillary mucinous neoplasm of the pancreas. World journal of gastroenterology : WJG. 2005; **11**(36): 5688-90.
273. Reid-Lombardo KM, Mathis KL, Wood CM, Harmsen WS, Sarr MG. Frequency of extrapancreatic neoplasms in intraductal papillary mucinous neoplasm of the pancreas: implications for management. Annals of surgery. 2010; **251**(1): 64-9.
274. Oh SJ, Lee SJ, Lee HY, Paik YH, Lee DK, Lee KS, et al. [Extrapancreatic tumors in intraductal papillary mucinous neoplasm of the pancreas]. The Korean journal of gastroenterology = Taehan Sohwagi Hakhoe chi. 2009; **54**(3): 162-6.
275. Baumgaertner I, Corcos O, Couvelard A, Sauvanet A, Rebours V, Vullierme MP, et al. Prevalence of extrapancreatic cancers in patients with histologically proven intraductal papillary mucinous neoplasms of the pancreas: a case-control study. The American journal of gastroenterology. 2008; **103**(11): 2878-82.
276. Choi MG, Kim SW, Han SS, Jang JY, Park YH. High incidence of extrapancreatic neoplasms in patients with intraductal papillary mucinous neoplasms. Arch Surg. 2006; **141**(1): 51-6; discussion 6.
277. Kent TS, Jr CM, Callery MP. Intraductal papillary mucinous neoplasm and the pancreatic incidentaloma. World journal of gastrointestinal surgery. 2010; **2**(10): 319-23.
278. Sawhney MS, Al-Bashir S, Cury MS, Brown A, Chuttani R, Pleskow DK, et al. International consensus guidelines for surgical resection of mucinous neoplasms cannot be applied to all cystic lesions of the pancreas. Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association. 2009; **7**(12): 1373-6.
279. Tanno S, Nakano Y, Nishikawa T, Nakamura K, Sasajima J, Minoguchi M, et al. Natural history of branch duct intraductal papillary-mucinous neoplasms of the pancreas without mural nodules: long-term follow-up results. Gut. 2008; **57**(3): 339-43.
280. Buscaglia JM, Shin EJ, Giday SA, Kapoor S, Dunbar KB, Eloubeidi MA, et al. Awareness of guidelines and trends in the management of suspected pancreatic cystic neoplasms: survey results among general gastroenterologists and EUS specialists. Gastrointestinal endoscopy. 2009; **69**(4): 813-20, quiz 20 e1-17.
281. Del Chiaro M, Verbeke C, Salvia R, Kloppel G, Werner J, McKay C, et al. European experts consensus statement on cystic tumours of the pancreas. Digestive and liver disease : official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver. 2013; **45**(9): 703-11.

282. Farnell MB. Surgical management of intraductal papillary mucinous neoplasm (IPMN) of the pancreas. Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract. 2008; **12**(3): 414-6.
283. Goh BK. Sendai Consensus Guidelines for Branch-duct IPMN: Guidelines Are Just Guidelines. Annals of surgery. 2015; **262**(2): e65.
284. Nguyen AH, Toste PA, Farrell JJ, Clerkin BM, Williams J, Muthusamy VR, et al. Current recommendations for surveillance and surgery of intraductal papillary mucinous neoplasms may overlook some patients with cancer. Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract. 2015; **19**(2): 258-65.
285. Vege SS, Ziring B, Jain R, Moayyedi P. American gastroenterological association institute guideline on the diagnosis and management of asymptomatic neoplastic pancreatic cysts. Gastroenterology. 2015; **148**(4): 819-22; quize12-3.
286. Kloppel G. Clinicopathologic view of intraductal papillary-mucinous tumor of the pancreas. Hepato-gastroenterology. 1998; **45**(24): 1981-5.
287. Traverso LW, Peralta EA, Ryan JA, Jr., Kozarek RA. Intraductal neoplasms of the pancreas. American journal of surgery. 1998; **175**(5): 426-32.
288. Sessa F, Solcia E, Capella C, Bonato M, Scarpa A, Zamboni G, et al. Intraductal papillary-mucinous tumours represent a distinct group of pancreatic neoplasms: an investigation of tumour cell differentiation and *KRAS*, p53 and c-erbB-2 abnormalities in 26 patients. Virchows Archiv : an international journal of pathology. 1994; **425**(4): 357-67.
289. Siech M, Tripp K, Schmidt-Rohlfing B, Mattfeldt T, Gorich J, Beger HG. Intraductal papillary mucinous tumor of the pancreas. American journal of surgery. 1999; **177**(2): 117-20.
290. Poultsides GA, Reddy S, Cameron JL, Hruban RH, Pawlik TM, Ahuja N, et al. Histopathologic basis for the favorable survival after resection of intraductal papillary mucinous neoplasm-associated invasive adenocarcinoma of the pancreas. Annals of surgery. 2010; **251**(3): 470-6.
291. Salvia R, Fernandez-del Castillo C, Bassi C, Thayer SP, Falconi M, Mantovani W, et al. Main-duct intraductal papillary mucinous neoplasms of the pancreas: clinical predictors of malignancy and long-term survival following resection. Annals of surgery. 2004; **239**(5): 678-85; discussion 85-7.
292. Xiao SY. Intraductal papillary mucinous neoplasm of the pancreas: an update. Scientifica. 2012; **2012**: 893632.
293. Pedrazzoli S, Sperti C, Pasquali C, Bissoli S, Chierichetti F. Comparison of International Consensus Guidelines versus 18-FDG PET in detecting malignancy of intraductal papillary mucinous neoplasms of the pancreas. Annals of surgery. 2011; **254**(6): 971-6.
294. Takanami K, Hiraide T, Tsuda M, Nakamura Y, Kaneta T, Takase K, et al. Additional value of FDG PET/CT to contrast-enhanced CT in the differentiation between benign and malignant intraductal papillary mucinous neoplasms of the pancreas with mural nodules. Annals of nuclear medicine. 2011; **25**(7): 501-10.
295. Takanami K, Yamada T, Tsuda M, Takase K, Ishida K, Nakamura Y, et al. Intraductal papillary mucininous neoplasm of the bile ducts: multimodality assessment with pathologic correlation. Abdominal imaging. 2011; **36**(4): 447-56.
296. Rastegar N, Matteoni-Athayde LG, Eng J, Takahashi N, Tamm EP, Mortele KJ, et al. Incremental value of secretin-enhanced magnetic resonance cholangiopancreatography in detecting ductal communication in a population with high prevalence of small pancreatic cysts. European journal of radiology. 2015; **84**(4): 575-80.
297. Fujino Y, Matsumoto I, Ueda T, Toyama H, Kuroda Y. Proposed new score predicting malignancy of intraductal papillary mucinous neoplasms of the pancreas. American journal of surgery. 2007; **194**(3): 304-7.
298. Fritz S, Hackert T, Hinz U, Hartwig W, Buchler MW, Werner J. Role of serum carbohydrate antigen 19-9 and carcinoembryonic antigen in distinguishing between benign and invasive intraductal papillary mucinous neoplasm of the pancreas. The British journal of surgery. 2011; **98**(1): 104-10.

299. Kawai M, Uchiyama K, Tani M, Onishi H, Kinoshita H, Ueno M, et al. Clinicopathological features of malignant intraductal papillary mucinous tumors of the pancreas: the differential diagnosis from benign entities. Arch Surg. 2004; **139**(2): 188-92.
300. Kanno A, Satoh K, Hirota M, Hamada S, Umino J, Itoh H, et al. Prediction of invasive carcinoma in branch type intraductal papillary mucinous neoplasms of the pancreas. Journal of gastroenterology. 2010; **45**(9): 952-9.
301. Doyle CJ, Yancey K, Pitt HA, Wang M, Bemis K, Yip-Schneider MT, et al. The proteome of normal pancreatic juice. Pancreas. 2012; **41**(2): 186-94.
302. Emerson RE, Randolph ML, Cramer HM. Endoscopic ultrasound-guided fine-needle aspiration cytology diagnosis of intraductal papillary mucinous neoplasm of the pancreas is highly predictive of pancreatic neoplasia. Diagnostic cytopathology. 2006; **34**(7): 457-62.
303. Jani N, Dewitt J, Eloubeidi M, Varadarajulu S, Appalaneni V, Hoffman B, et al. Endoscopic ultrasound-guided fine-needle aspiration for diagnosis of solid pseudopapillary tumors of the pancreas: a multicenter experience. Endoscopy. 2008; **40**(3): 200-3.
304. Burford H, Baloch Z, Liu X, Jhala D, Siegal GP, Jhala N. E-cadherin/beta-catenin and CD10: a limited immunohistochemical panel to distinguish pancreatic endocrine neoplasm from solid pseudopapillary neoplasm of the pancreas on endoscopic ultrasound-guided fine-needle aspirates of the pancreas. American journal of clinical pathology. 2009; **132**(6): 831-9.
305. Layfield LJ, Cramer H. Fine-needle aspiration cytology of intraductal papillary-mucinous tumors: a retrospective analysis. Diagnostic cytopathology. 2005; **32**(1): 16-20.
306. Rockacy M, Khalid A. Update on pancreatic cyst fluid analysis. Annals of gastroenterology : quarterly publication of the Hellenic Society of Gastroenterology. 2013; **26**(2): 122-7.
307. Rockacy MJ, Zahid M, McGrath KM, Fasanella KE, Khalid A. Association between KRAS mutation, detected in pancreatic cyst fluid, and long-term outcomes of patients. Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association. 2013; **11**(4): 425-9.
308. Kamoshida S, Tsutsumi Y. Expression of MUC-1 glycoprotein in plasma cells, follicular dendritic cells, myofibroblasts and perineurial cells: immunohistochemical analysis using three monoclonal antibodies. Pathology international. 1998; **48**(10): 776-85.
309. Pantano F, Baldi A, Santini D, Vincenzi B, Borzomati D, Vecchio FM, et al. MUC2 but not MUC5 expression correlates with prognosis in radically resected pancreatic cancer patients. Cancer biology & therapy. 2009; **8**(11): 996-9.
310. Rassidakis GZ, Goy A, Medeiros LJ, Jiang Y, Thomaides A, Remache Y, et al. Prognostic Significance of MUC-1 Expression in Systemic Anaplastic Large Cell Lymphoma. Clinical Cancer Research. 2003; **9**(6): 2213-20.
311. Leroy X, Zerimech F, Zini L, Copin MC, Buisine MP, Gosselin B, et al. MUC1 expression is correlated with nuclear grade and tumor progression in pT1 renal clear cell carcinoma. American journal of clinical pathology. 2002; **118**(1): 47-51.
312. Sinn BV, von Minckwitz G, Denkert C, Eidtmann H, Darb-Esfahani S, Tesch H, et al. Evaluation of Mucin-1 protein and mRNA expression as prognostic and predictive markers after neoadjuvant chemotherapy for breast cancer. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2013; **24**(9): 2316-24.
313. Engelstaedter V, Heublein S, Schumacher AL, Lenhard M, Engelstaedter H, Andergassen U, et al. Mucin-1 and its relation to grade, stage and survival in ovarian carcinoma patients. BMC cancer. 2012; **12**: 600.
314. Park SY, Roh SJ, Kim YN, Kim SZ, Park HS, Jang KY, et al. Expression of MUC1, MUC2, MUC5AC and MUC6 in cholangiocarcinoma: prognostic impact. Oncology reports. 2009; **22**(3): 649-57.
315. You JF, Hsieh LL, Changchien CR, Chen JS, Chen JR, Chiang JM, et al. Inverse effects of mucin on survival of matched hereditary nonpolyposis colorectal cancer and sporadic colorectal cancer patients. Clinical cancer research : an official journal of the American Association for Cancer Research. 2006; **12**(14 Pt 1): 4244-50.

316. Li XH, Zheng HC, Wang ZG, Takahashi H, Yang XH, Guan YF, et al. The clinicopathological and prognostic significance of MUC-1 expression in Japanese gastric carcinomas: an immunohistochemical study of tissue microarrays. Anticancer research. 2008; **28**(2A): 1061-7.
317. Utsunomiya T, Yonezawa S, Sakamoto H, Kitamura H, Hokita S, Aiko T, et al. Expression of MUC1 and MUC2 mucins in gastric carcinomas: its relationship with the prognosis of the patients. Clinical cancer research : an official journal of the American Association for Cancer Research. 1998; **4**(11): 2605-14.
318. Baldus SE, Monig SP, Hanisch FG, Zirbes TK, Flucke U, Oelert S, et al. Comparative evaluation of the prognostic value of MUC1, MUC2, sialyl-Lewis(a) and sialyl-Lewis(x) antigens in colorectal adenocarcinoma. Histopathology. 2002; **40**(5): 440-9.
319. Manne U, Weiss HL, Grizzle WE. Racial differences in the prognostic usefulness of MUC1 and MUC2 in colorectal adenocarcinomas. Clinical cancer research : an official journal of the American Association for Cancer Research. 2000; **6**(10): 4017-25.
320. Pfeifer GP, Denissenko MF, Olivier M, Tretyakova N, Hecht SS, Hainaut P. Tobacco smoke carcinogens, DNA damage and p53 mutations in smoking-associated cancers. Oncogene. 2002; **21**(48): 7435-51.
321. LaRue H, Allard P, Simoneau M, Normand C, Pfister C, Moore L, et al. P53 point mutations in initial superficial bladder cancer occur only in tumors from current or recent cigarette smokers. Carcinogenesis. 2000; **21**(1): 101-6.
322. Malakar M, Devi KR, Phukan RK, Kaur T, Deka M, Puia L, et al. p53 codon 72 polymorphism interactions with dietary and tobacco related habits and risk of stomach cancer in Mizoram, India. Asian Pacific journal of cancer prevention : APJCP. 2014; **15**(2): 717-23.
323. Rezaee N, Khalifian S, Cameron JL, Pawlik TM, Hruban RH, Fishman EK, et al. Smoking is not associated with severe dysplasia or invasive carcinoma in resected intraductal papillary mucinous neoplasms. Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract. 2015; **19**(4): 656-65.
324. De Re V, Carbone A, De Vita S, Gloghini A, Maestro R, Gasparotto D, et al. p53 protein over-expression and p53 gene abnormalities in HIV-1-related non-Hodgkin's lymphomas. International journal of cancer Journal international du cancer. 1994; **56**(5): 662-7.
325. Barton CM, Staddon SL, Hughes CM, Hall PA, O'Sullivan C, Kloppel G, et al. Abnormalities of the p53 tumour suppressor gene in human pancreatic cancer. British journal of cancer. 1991; **64**(6): 1076-82.
326. Du M, Peng H, Singh N, Isaacson PG, Pan L. The accumulation of p53 abnormalities is associated with progression of mucosa-associated lymphoid tissue lymphoma. Blood. 1995; **86**(12): 4587-93.
327. Sturm PD, Baas IO, Clement MJ, Nakeeb A, Johan G, Offerhaus A, et al. Alterations of the p53 tumor-suppressor gene and *KRAS* oncogene in perihilar cholangiocarcinomas from a high-incidence area. International journal of cancer Journal international du cancer. 1998; **78**(6): 695-8.
328. Makower D, Rozenblit A, Kaufman H, Edelman M, Lane ME, Zwiebel J, et al. Phase II clinical trial of intralesional administration of the oncolytic adenovirus ONYX-015 in patients with hepatobiliary tumors with correlative p53 studies. Clinical cancer research : an official journal of the American Association for Cancer Research. 2003; **9**(2): 693-702.
329. Shiraishi K, Kato S, Han SY, Liu W, Otsuka K, Sakayori M, et al. Isolation of temperature-sensitive p53 mutations from a comprehensive missense mutation library. The Journal of biological chemistry. 2004; **279**(1): 348-55.
330. Shaw P, Tardy S, Benito E, Obrador A, Costa J. Occurrence of Ki-ras and p53 mutations in primary colorectal tumors. Oncogene. 1991; **6**(11): 2121-8.
331. Scarpa A, Capelli P, Mukai K, Zamboni G, Oda T, Iacono C, et al. Pancreatic adenocarcinomas frequently show p53 gene mutations. The American journal of pathology. 1993; **142**(5): 1534-43.

332. Weyrer K, Feichtinger H, Haun M, Weiss G, Ofner D, Weger AR, et al. p53, Ki-ras, and DNA ploidy in human pancreatic ductal adenocarcinomas. Laboratory investigation; a journal of technical methods and pathology. 1996; **74**(1): 279-89.
333. Moore PS, Orlandini S, Zamboni G, Capelli P, Rigaud G, Falconi M, et al. Pancreatic tumours: molecular pathways implicated in ductal cancer are involved in ampullary but not in exocrine nonductal or endocrine tumorigenesis. British journal of cancer. 2001; **84**(2): 253-62.
334. Ong CK, Subimerb C, Pairojkul C, Wongkham S, Cutcutache I, Yu W, et al. Exome sequencing of liver fluke-associated cholangiocarcinoma. Nature genetics. 2012; **44**(6): 690-3.
335. Tang Y, Zhao W, Chen Y, Zhao Y, Gu W. Acetylation Is Indispensable for p53 Activation. Cell. 2008; **133**(4): 612-26. doi:10.1016/j.cell.2008.03.025.
336. Wen WH, Reles A, Runnebaum IB, Sullivan-Halley J, Bernstein L, Jones LA, et al. p53 mutations and expression in ovarian cancers: correlation with overall survival. International journal of gynecological pathology : official journal of the International Society of Gynecological Pathologists. 1999; **18**(1): 29-41.
337. Matozaki T, Sakamoto C, Suzuki T, Matsuda K, Uchida T, Nakano O, et al. p53 gene mutations in human gastric cancer: wild-type p53 but not mutant p53 suppresses growth of human gastric cancer cells. Cancer research. 1992; **52**(16): 4335-41.
338. Bamford S, Dawson E, Forbes S, Clements J, Pettett R, Dogan A, et al. The COSMIC (Catalogue of Somatic Mutations in Cancer) database and website. British journal of cancer. 2004; **91**(2): 355-8.
339. Mashima T, Oh-hara T, Sato S, Mochizuki M, Sugimoto Y, Yamazaki K, et al. p53-defective tumors with a functional apoptosome-mediated pathway: a new therapeutic target. Journal of the National Cancer Institute. 2005; **97**(10): 765-77.
340. Tomoda K, Takahashi N, Hibi Y, Asamitsu K, Ishida H, Kondo T, et al. Molecular docking analysis of the protein-protein interaction between RelA-associated inhibitor and tumor suppressor protein p53 and its inhibitory effect on p53 action. Cancer science. 2008; **99**(3): 615-22.
341. Colomer A, Erill N, Verdu M, Roman R, Vidal A, Cordon-Cardo C, et al. Lack of p53 nuclear immunostaining is not indicative of absence of *TP53* gene mutations in colorectal adenocarcinomas. Applied immunohistochemistry & molecular morphology : AIMM / official publication of the Society for Applied Immunohistochemistry. 2003; **11**(2): 130-7.
342. O'Connor PM, Jackman J, Bae I, Myers TG, Fan S, Mutoh M, et al. Characterization of the p53 tumor suppressor pathway in cell lines of the National Cancer Institute anticancer drug screen and correlations with the growth-inhibitory potency of 123 anticancer agents. Cancer research. 1997; **57**(19): 4285-300.
343. Cai DW, Mukhopadhyay T, Roth JA. Suppression of lung cancer cell growth by ribozyme-mediated modification of p53 pre-mRNA. Cancer gene therapy. 1995; **2**(3): 199-205.
344. Shimizu H, Burch LR, Smith AJ, Dornan D, Wallace M, Ball KL, et al. The conformationally flexible S9-S10 linker region in the core domain of p53 contains a novel MDM2 binding site whose mutation increases ubiquitination of p53 in vivo. The Journal of biological chemistry. 2002; **277**(32): 28446-58.
345. Chung KY, Mukhopadhyay T, Kim J, Casson A, Ro JY, Goepfert H, et al. Discordant p53 gene mutations in primary head and neck cancers and corresponding second primary cancers of the upper aerodigestive tract. Cancer research. 1993; **53**(7): 1676-83.
346. Chiba I, Takahashi T, Nau MM, D'Amico D, Curiel DT, Mitsudomi T, et al. Mutations in the p53 gene are frequent in primary, resected non-small cell lung cancer. Lung Cancer Study Group. Oncogene. 1990; **5**(10): 1603-10.
347. Ruggeri B, Zhang SY, Klein-Szanto AJ. Molecular and cytogenetic alterations in human pancreatic cancer: the role of tumor suppressor genes. Progress in clinical and biological research. 1992; **376**: 245-60.
348. Berrozpe G, Schaeffer J, Peinado MA, Real FX, Perucho M. Comparative analysis of mutations in the p53 and *KRAS* genes in pancreatic cancer. International journal of cancer Journal international du cancer. 1994; **58**(2): 185-91.

349. Pellegata NS, Sessa F, Renault B, Bonato M, Leone BE, Solcia E, et al. *KRAS* and p53 gene mutations in pancreatic cancer: ductal and nonductal tumors progress through different genetic lesions. Cancer research. 1994; **54**(6): 1556-60.
350. Hoshi T, Imai M, Ogawa K. Frequent *KRAS* mutations and absence of p53 mutations in mucin-producing tumors of the pancreas. Journal of surgical oncology. 1994; **55**(2): 84-91.
351. Nakamori S, Yashima K, Murakami Y, Ishikawa O, Ohigashi H, Imaoka S, et al. Association of p53 gene mutations with short survival in pancreatic adenocarcinoma. Japanese journal of cancer research : Gann. 1995; **86**(2): 174-81.
352. Chen J, Millar WJ. Are recent cohorts healthier than their predecessors? Health reports / Statistics Canada, Canadian Centre for Health Information = Rapports sur la sante / Statistique Canada, Centre canadien d'information sur la sante. 2000; **11**(4): 9-23 (Eng); 9-6 (Fre).
353. Ruggeri BA, Huang L, Berger D, Chang H, Klein-Szanto AJ, Goodrow T, et al. Molecular pathology of primary and metastatic ductal pancreatic lesions: analyses of mutations and expression of the p53, mdm-2, and p21/WAF-1 genes in sporadic and familial lesions. Cancer. 1997; **79**(4): 700-16.
354. Rozenblum E, Schutte M, Goggins M, Hahn SA, Panzer S, Zahurak M, et al. Tumor-suppressive pathways in pancreatic carcinoma. Cancer research. 1997; **57**(9): 1731-4.
355. Iwao T, Hanada K, Tsuchida A, Hirata M, Eguchi N, Kajiyama G. The establishment of a preoperative diagnosis of pancreatic carcinoma using cell specimens from pancreatic duct brushing with special attention to p53 mutations. Cancer. 1998; **82**(8): 1487-94.
356. Sorio C, Bonora A, Orlandini S, Moore PS, Capelli P, Cristofori P, et al. Successful xenografting of cryopreserved primary pancreatic cancers. Virchows Archiv : an international journal of pathology. 2001; **438**(2): 154-8.
357. Kang S, Duan LH, Zhang JH, Guo W, Wang N, Li Y. [Association of p53 gene polymorphism with susceptibility to ovarian cancer]. Zhonghua fu chan ke za zhi. 2004; **39**(11): 754-8.
358. Bian Y, Matsubayashi H, Li CP, Abe T, Canto M, Murphy KM, et al. Detecting low-abundance p16 and p53 mutations in pancreatic juice using a novel assay: heteroduplex analysis of limiting dilution PCRs. Cancer biology & therapy. 2006; **5**(10): 1392-9.
359. Jones S, Zhang X, Parsons DW, Lin JC, Leary RJ, Angenendt P, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science. 2008; **321**(5897): 1801-6.
360. Stindt MH, Muller PA, Ludwig RL, Kehrloesser S, Dotsch V, Vousden KH. Functional interplay between MDM2, p63/p73 and mutant p53. Oncogene. 2014.
361. Dong P, Tada M, Hamada J, Nakamura A, Moriuchi T, Sakuragi N. p53 dominant-negative mutant R273H promotes invasion and migration of human endometrial cancer HHUA cells. Clinical & experimental metastasis. 2007; **24**(6): 471-83.
362. Soto AM, Sonnenschein C. The tissue organization field theory of cancer: a testable replacement for the somatic mutation theory. BioEssays : news and reviews in molecular, cellular and developmental biology. 2011; **33**(5): 332-40.
363. Kanda M, Knight S, Topazian M, Syngal S, Farrell J, Lee J, et al. Mutant GNAS detected in duodenal collections of secretin-stimulated pancreatic juice indicates the presence or emergence of pancreatic cysts. Gut. 2012.

Appendix A: Concentration of DNA obtained from each Heidelberg sample.

	Concentration of DNA (ng/uL)			Concentration of DNA (ng/uL)	
CYST 0-	Eluent 1	Eluent 2	CYST 0-	Eluent 1	Eluent 2
1	119	165	37	137.4	113.4
2	2.1	2.9	38	46.8	33.7
3	15.6	9	39	68.4	475.6
4	10.6	7	40	73.1	71.7
5	28.1	32.5	41	239.8	109.2
6	15.9	21.3	42	136	240.8
7	6.8	5.8	43	165.9	53.9
8	30.6	31.7	44	13.9	44.4
9	81	51.2	45	22.2	21.4
10	125.6	76.7	46	8.7	19.8
11	109.5	43.2	47	14.6	18.4
12	119.7	73.5	48	15.9	17
13	38.7	26.7	49	33.2	42.3
14	6.9	3.2	50	32.2	43.7
15	36.1	44.6	51	55.9	168.1
16	11.8	12.4	52	37.5	42.3
17	34.5	134.6	53	74.5	97
18	39.3	1008.2	54	36.2	31.4
19	4.1	4.7	55	3.2	1.4
20	7.3	11.7	56	16.2	10.6
21	24.1	14.6	57	69	37.2
22	745	171.8	58	75.1	153.9
23	87.3	126.3	59	55.5	69.6
24	43.4	21.5	60	57	77.3
25	71.4	37.8	61	12.4	9.3
26	15.3	10	62	43.3	61
27	348.5	33.9	63	38.5	30.3
28	38.5	32.3	64	79.5	144.1
29	91	68.2	65	59.7	76.6
30	56.2	32.7	66	158	570
31	1177.9	406.6	67	68.2	109.1
32	68	125.9	68	128.6	130.5
33	71	53.7	69	39.1	29.7
34	72.4	96.5	70	28.9	35.4
35	43.2	55.2	71	6.1	7.6
36	54.1	70.8	72	53.6	45.4
			73	11.3	13.9

Appendix B: *KRAS* primers used to confirm samples were at 10G.

Forward control *KRAS* primer (5'TGACTGAATATAAACTTGTGGTAGTTGGCG3') and

Common Reverse *KRAS* primer (5'CTCATGAAAATGGTCAGAGAAACCTTTATC3').

Alanine (5'TATCGTCAAGGCACTCTTGCCTACGCCTG 3');

Valine (5'TATCGTCAAGGCACTCTTGCCTACGCCTA 3');

12 Aspartate (5'TATCGTCAAGGCACTCTTGCCTACGCCTT 3');

Serine (5'CTGAATATAAACTTGTGGTAGTTGGAGCCA 3');

Arginine (5'CTGAATATAAACTTGTGGTAGTTGGAGTTC 3');

Cystine (5'CTGAATATAAACTTGTGGTAGTTGGAGCAT 3').

Use the appropriate common primer pair: either Common Forward (5'GTACTGGTGGAGTATTTGATAGTGTATTAACC3') or

Common Reverse (5'CTCATGAAAATGGTCAGAGAAACCTTTATC3').

The PCR programme used for standard *KRAS* PCR (hot start, melting temp, annealing and binding temp etc.)

Pre-Incubation:

95^{o}C for 10 minutes

Amplification cycles (at least 60) consisting of:

95^{o}C for 10 seconds

61 ^{o}C for 5 seconds

72 ^{o}C for 5 seconds with a single fluorescence measurement at the end

Melting Curve:

95 ^{o}C for 5 seconds

65 ^{o}C for 1 minute

Then a slow ramp to 98 ^{o}C over a 10 minute period with continuous fluorescence measurement

Cooling:

40 ^{o}C for 10 seconds then cooled to 2 ^{o}C which was held.

Appendix C: List of individually created barcoded primers used for Ion Torrent.

Oligo name	Oligo sequence (5' to 3')	Synthesis scale
Exon 5 F1 A-1.1	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAAACAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 A-1.2	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAACCAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 A-1.3	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAAGCAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 A-1.4	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAATCAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 A-1.5	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACACAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 A-1.6	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACCCAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 A-1.7	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACGCAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 A-1.8	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACTCAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 A-1.9	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGACAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 A-1.10	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGCCAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 P-1.1	CCT CTC TAT GGG CAG TCG GTG ATAAAC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F1 P-1.2	CCT CTC TAT GGG CAG TCG GTG ATAACC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F1 P-1.3	CCT CTC TAT GGG CAG TCG GTG ATAAGC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F1 P-1.4	CCT CTC TAT GGG CAG TCG GTG ATAATC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F1 P-1.5	CCT CTC TAT GGG CAG TCG GTG ATACAC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F1 P-1.6	CCT CTC TAT GGG CAG TCG GTG ATACCC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F1 P-1.7	CCT CTC TAT GGG CAG TCG GTG ATACGC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F1 P-1.8	CCT CTC TAT GGG CAG TCG GTG ATACTC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F1 P-1.9	CCT CTC TAT GGG CAG TCG GTG ATAGAC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F1 P-1.10	CCT CTC TAT GGG CAG TCG GTG ATAGCC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F2 A-1.1	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAAAAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 A-1.2	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAACAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 A-1.3	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAAGAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 A-1.4	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAATAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 A-1.5	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACAAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 A-1.6	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACCAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 A-1.7	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACGAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 A-1.8	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACTAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 A-1.9	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGAAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 A-1.10	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGCAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 P-1.1	CCT CTC TAT GGG CAG TCG GTG ATAAAA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F2 P-1.2	CCT CTC TAT GGG CAG TCG GTG ATAACA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F2 P-1.3	CCT CTC TAT GGG CAG TCG GTG ATAAGA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F2 P-1.4	CCT CTC TAT GGG CAG TCG GTG ATAATA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F2 P-1.5	CCT CTC TAT GGG CAG TCG GTG ATACAA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F2 P-1.6	CCT CTC TAT GGG CAG TCG GTG ATACCA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F2 P-1.7	CCT CTC TAT GGG CAG TCG GTG ATACGA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F2 P-1.8	CCT CTC TAT GGG CAG TCG GTG ATACTA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F2 P-1.9	CCT CTC TAT GGG CAG TCG GTG ATAGAA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F2 P-1.10	CCT CTC TAT GGG CAG TCG GTG ATAGCA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F3 A-1.1	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAAATGG CCA TCT ACA AGC AGT CA	200N
Exon 5 F3 A-1.2	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAACTGG CCA TCT ACA AGC AGT CA	200N
Exon 5 F3 A-1.3	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAAGTGG CCA TCT ACA AGC AGT CA	200N
Exon 5 F3 A-1.4	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAATTGG CCA TCT ACA AGC AGT CA	200N
Exon 5 F3 A-1.5	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACATGG CCA TCT ACA AGC AGT CA	200N

Exon 5 F3 A-1.6	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACCTGG CCA TCT ACA AGC AGT CA	200N
Exon 5 F3 A-1.7	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACGTGG CCA TCT ACA AGC AGT CA	200N
Exon 5 F3 A-1.8	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACTTGG CCA TCT ACA AGC AGT CA	200N
Exon 5 F3 A-1.9	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGATGG CCA TCT ACA AGC AGT CA	200N
Exon 5 F3 A-1.10	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGCTGG CCA TCT ACA AGC AGT CA	200N
Exon 5 F3 P-1.1	CCT CTC TAT GGG CAG TCG GTG ATAAAT GGC CAT CTA CAA GCA GTC A	200N
Exon 5 F3 P-1.2	CCT CTC TAT GGG CAG TCG GTG ATAACT GGC CAT CTA CAA GCA GTC A	200N
Exon 5 F3 P-1.3	CCT CTC TAT GGG CAG TCG GTG ATAAGT GGC CAT CTA CAA GCA GTC A	200N
Exon 5 F3 P-1.4	CCT CTC TAT GGG CAG TCG GTG ATAATT GGC CAT CTA CAA GCA GTC A	200N
Exon 5 F3 P-1.5	CCT CTC TAT GGG CAG TCG GTG ATACAT GGC CAT CTA CAA GCA GTC A	200N
Exon 5 F3 P-1.6	CCT CTC TAT GGG CAG TCG GTG ATACCT GGC CAT CTA CAA GCA GTC A	200N
Exon 5 F3 P-1.7	CCT CTC TAT GGG CAG TCG GTG ATACGT GGC CAT CTA CAA GCA GTC A	200N
Exon 5 F3 P-1.8	CCT CTC TAT GGG CAG TCG GTG ATACTT GGC CAT CTA CAA GCA GTC A	200N
Exon 5 F3 P-1.9	CCT CTC TAT GGG CAG TCG GTG ATAGAT GGC CAT CTA CAA GCA GTC A	200N
Exon 5 F3 P-1.10	CCT CTC TAT GGG CAG TCG GTG ATAGCT GGC CAT CTA CAA GCA GTC A	200N
Exon 6 F A-1.1	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAAACAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F A-1.2	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAACCAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F A-1.3	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAAGCAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F A-1.4	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAATCAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F A-1.5	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACACAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F A-1.6	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACCCAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F A-1.7	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACGCAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F A-1.8	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACTCAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F A-1.9	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGACAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F A-1.10	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGCCAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F P-1.1	CCT CTC TAT GGG CAG TCG GTG ATAAAC AGG CCT CTG ATT CCT CAC T	200N
Exon 6 F P-1.2	CCT CTC TAT GGG CAG TCG GTG ATAACC AGG CCT CTG ATT CCT CAC T	200N
Exon 6 F P-1.3	CCT CTC TAT GGG CAG TCG GTG ATAAGC AGG CCT CTG ATT CCT CAC T	200N
Exon 6 F P-1.4	CCT CTC TAT GGG CAG TCG GTG ATAATC AGG CCT CTG ATT CCT CAC T	200N
Exon 6 F P-1.5	CCT CTC TAT GGG CAG TCG GTG ATACAC AGG CCT CTG ATT CCT CAC T	200N
Exon 6 F P-1.6	CCT CTC TAT GGG CAG TCG GTG ATACCC AGG CCT CTG ATT CCT CAC T	200N
Exon 6 F P-1.7	CCT CTC TAT GGG CAG TCG GTG ATACGC AGG CCT CTG ATT CCT CAC T	200N
Exon 6 F P-1.8	CCT CTC TAT GGG CAG TCG GTG ATACTC AGG CCT CTG ATT CCT CAC T	200N
Exon 6 F P-1.9	CCT CTC TAT GGG CAG TCG GTG ATAGAC AGG CCT CTG ATT CCT CAC T	200N
Exon 6 F P-1.10	CCT CTC TAT GGG CAG TCG GTG ATAGCC AGG CCT CTG ATT CCT CAC T	200N
Exon 7 F1 A-1.1	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAAACCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 A-1.2	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAACCCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 A-1.3	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAAGCCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 A-1.4	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAATCCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 A-1.5	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACACCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 A-1.6	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACCCCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 A-1.7	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACGCCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 A-1.8	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACTCCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 A-1.9	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGACCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 A-1.10	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGCCCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 P-1.1	CCT CTC TAT GGG CAG TCG GTG ATAAAC CTG CTT GCC ACA GGT CT	200N
Exon 7 F1 P-1.2	CCT CTC TAT GGG CAG TCG GTG ATAACC CTG CTT GCC ACA GGT CT	200N
Exon 7 F1 P-1.3	CCT CTC TAT GGG CAG TCG GTG ATAAGC CTG CTT GCC ACA GGT CT	200N
Exon 7 F1 P-1.4	CCT CTC TAT GGG CAG TCG GTG ATAATC CTG CTT GCC ACA GGT CT	200N
Exon 7 F1 P-1.5	CCT CTC TAT GGG CAG TCG GTG ATACAC CTG CTT GCC ACA GGT CT	200N
Exon 7 F1 P-1.6	CCT CTC TAT GGG CAG TCG GTG ATACCC CTG CTT GCC ACA GGT CT	200N
Exon 7 F1 P-1.7	CCT CTC TAT GGG CAG TCG GTG ATACGC CTG CTT GCC ACA GGT CT	200N

Exon 7 F1 P-1.8	CCT CTC TAT GGG CAG TCG GTG ATACTC CTG CTT GCC ACA GGT CT	200N
Exon 7 F1 P-1.9	CCT CTC TAT GGG CAG TCG GTG ATAGAC CTG CTT GCC ACA GGT CT	200N
Exon 7 F1 P-1.10	CCT CTC TAT GGG CAG TCG GTG ATAGCC CTG CTT GCC ACA GGT CT	200N
Exon 7 F2 A-1.1	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAAACCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 A-1.2	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAACCCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 A-1.3	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAAGCCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 A-1.4	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAATCCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 A-1.5	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACACCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 A-1.6	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACCCCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 A-1.7	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACGCCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 A-1.8	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACTCCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 A-1.9	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGACCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 A-1.10	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGCCCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 P-1.1	CCT CTC TAT GGG CAG TCG GTG ATAAAC CTC ACC ATC ATC ACA CTG G	200N
Exon 7 F2 P-1.2	CCT CTC TAT GGG CAG TCG GTG ATAACC CTC ACC ATC ATC ACA CTG G	200N
Exon 7 F2 P-1.3	CCT CTC TAT GGG CAG TCG GTG ATAAGC CTC ACC ATC ATC ACA CTG G	200N
Exon 7 F2 P-1.4	CCT CTC TAT GGG CAG TCG GTG ATAATC CTC ACC ATC ATC ACA CTG G	200N
Exon 7 F2 P-1.5	CCT CTC TAT GGG CAG TCG GTG ATACAC CTC ACC ATC ATC ACA CTG G	200N
Exon 7 F2 P-1.6	CCT CTC TAT GGG CAG TCG GTG ATACCC CTC ACC ATC ATC ACA CTG G	200N
Exon 7 F2 P-1.7	CCT CTC TAT GGG CAG TCG GTG ATACGC CTC ACC ATC ATC ACA CTG G	200N
Exon 7 F2 P-1.8	CCT CTC TAT GGG CAG TCG GTG ATACTC CTC ACC ATC ATC ACA CTG G	200N
Exon 7 F2 P-1.9	CCT CTC TAT GGG CAG TCG GTG ATAGAC CTC ACC ATC ATC ACA CTG G	200N
Exon 7 F2 P-1.10	CCT CTC TAT GGG CAG TCG GTG ATAGCC CTC ACC ATC ATC ACA CTG G	200N
Exon 8 F1 A-1.1	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAAATGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 A-1.2	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAACTGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 A-1.3	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAAGTGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 A-1.4	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAATTGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 A-1.5	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACATGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 A-1.6	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACCTGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 A-1.7	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACGTGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 A-1.8	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACTTGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 A-1.9	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGATGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 A-1.10	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGCTGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 P-1.1	CCT CTC TAT GGG CAG TCG GTG ATAAAT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F1 P-1.2	CCT CTC TAT GGG CAG TCG GTG ATAACT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F1 P-1.3	CCT CTC TAT GGG CAG TCG GTG ATAAGT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F1 P-1.4	CCT CTC TAT GGG CAG TCG GTG ATAATT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F1 P-1.5	CCT CTC TAT GGG CAG TCG GTG ATACAT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F1 P-1.6	CCT CTC TAT GGG CAG TCG GTG ATACCT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F1 P-1.7	CCT CTC TAT GGG CAG TCG GTG ATACGT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F1 P-1.8	CCT CTC TAT GGG CAG TCG GTG ATACTT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F1 P-1.9	CCT CTC TAT GGG CAG TCG GTG ATAGAT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F1 P-1.10	CCT CTC TAT GGG CAG TCG GTG ATAGCT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F2 A-1.1	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAAAACT GGG ACG GAA CAG CTT TG	200N
Exon 8 F2 A-1.2	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAACACT GGG ACG GAA CAG CTT TG	200N
Exon 8 F2 A-1.3	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAAGACT GGG ACG GAA CAG CTT TG	200N
Exon 8 F2 A-1.4	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAATACT GGG ACG GAA CAG CTT TG	200N
Exon 8 F2 A-1.5	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACAACT GGG ACG GAA CAG CTT TG	200N
Exon 8 F2 A-1.6	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACCACT GGG ACG GAA CAG CTT TG	200N
Exon 8 F2 A-1.7	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACGACT GGG ACG GAA CAG CTT TG	200N
Exon 8 F2 A-1.8	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGACTACT GGG ACG GAA CAG CTT TG	200N
Exon 8 F2 A-1.9	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGAACT GGG ACG GAA CAG CTT TG	200N

Exon 8 F2 A-1.10	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGCACT GGG ACG GAA CAG CTT TG	200N
Exon 8 F2 P-1.1	CCT CTC TAT GGG CAG TCG GTG ATAAAA CTG GGA CGG AAC AGC TTT G	200N
Exon 8 F2 P-1.2	CCT CTC TAT GGG CAG TCG GTG ATAACA CTG GGA CGG AAC AGC TTT G	200N
Exon 8 F2 P-1.3	CCT CTC TAT GGG CAG TCG GTG ATAAGA CTG GGA CGG AAC AGC TTT G	200N
Exon 8 F2 P-1.4	CCT CTC TAT GGG CAG TCG GTG ATAATA CTG GGA CGG AAC AGC TTT G	200N
Exon 8 F2 P-1.5	CCT CTC TAT GGG CAG TCG GTG ATACAA CTG GGA CGG AAC AGC TTT G	200N
Exon 8 F2 P-1.6	CCT CTC TAT GGG CAG TCG GTG ATACCA CTG GGA CGG AAC AGC TTT G	200N
Exon 8 F2 P-1.7	CCT CTC TAT GGG CAG TCG GTG ATACGA CTG GGA CGG AAC AGC TTT G	200N
Exon 8 F2 P-1.8	CCT CTC TAT GGG CAG TCG GTG ATACTA CTG GGA CGG AAC AGC TTT G	200N
Exon 8 F2 P-1.9	CCT CTC TAT GGG CAG TCG GTG ATAGAA CTG GGA CGG AAC AGC TTT G	200N
Exon 8 F2 P-1.10	CCT CTC TAT GGG CAG TCG GTG ATAGCA CTG GGA CGG AAC AGC TTT G	200N
Exon 5 F1 A-2.1	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGGCAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 A-2.2	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGTCAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 A-2.3	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATACAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 A-2.4	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATCCAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 A-2.5	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATGCAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 A-2.6	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATTCAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 A-2.7	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCAACAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 A-2.8	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCACCAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 A-2.9	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCAGCAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 A-2.10	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCATCAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 P-2.1	CCT CTC TAT GGG CAG TCG GTG ATAGGC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F1 P-2.2	CCT CTC TAT GGG CAG TCG GTG ATAGTC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F1 P-2.3	CCT CTC TAT GGG CAG TCG GTG ATATAC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F1 P-2.4	CCT CTC TAT GGG CAG TCG GTG ATATCC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F1 P-2.5	CCT CTC TAT GGG CAG TCG GTG ATATGC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F1 P-2.6	CCT CTC TAT GGG CAG TCG GTG ATATTC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F1 P-2.7	CCT CTC TAT GGG CAG TCG GTG ATCAAC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F1 P-2.8	CCT CTC TAT GGG CAG TCG GTG ATCACC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F1 P-2.9	CCT CTC TAT GGG CAG TCG GTG ATCAGC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F1 P-2.10	CCT CTC TAT GGG CAG TCG GTG ATCATC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F2 A-2.1	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGGAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 A-2.2	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGTAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 A-2.3	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATAAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 A-2.4	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATCAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 A-2.5	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATGAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 A-2.6	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATTAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 A-2.7	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCAAAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 A-2.8	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCACAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 A-2.9	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCAGAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 A-2.10	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCATAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 P-2.1	CCT CTC TAT GGG CAG TCG GTG ATAGGA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F2 P-2.2	CCT CTC TAT GGG CAG TCG GTG ATAGTA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F2 P-2.3	CCT CTC TAT GGG CAG TCG GTG ATATAA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F2 P-2.4	CCT CTC TAT GGG CAG TCG GTG ATATCA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F2 P-2.5	CCT CTC TAT GGG CAG TCG GTG ATATGA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F2 P-2.6	CCT CTC TAT GGG CAG TCG GTG ATATTA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F2 P-2.7	CCT CTC TAT GGG CAG TCG GTG ATCAAA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F2 P-2.8	CCT CTC TAT GGG CAG TCG GTG ATCACA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F2 P-2.9	CCT CTC TAT GGG CAG TCG GTG ATCAGA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F2 P-2.10	CCT CTC TAT GGG CAG TCG GTG ATCATA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F3 A-2.1	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGGTGG CCA TCT ACA AGC AGT CA	200N

Exon 5 F3 A-2.2	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGTTGG CCA TCT ACA AGC AGT CA	200N
Exon 5 F3 A-2.3	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATATGG CCA TCT ACA AGC AGT CA	200N
Exon 5 F3 A-2.4	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATCTGG CCA TCT ACA AGC AGT CA	200N
Exon 5 F3 A-2.5	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATGTGG CCA TCT ACA AGC AGT CA	200N
Exon 5 F3 A-2.6	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATTTGG CCA TCT ACA AGC AGT CA	200N
Exon 5 F3 A-2.7	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCAATGG CCA TCT ACA AGC AGT CA	200N
Exon 5 F3 A-2.8	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCACTGG CCA TCT ACA AGC AGT CA	200N
Exon 5 F3 A-2.9	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCAGTGG CCA TCT ACA AGC AGT CA	200N
Exon 5 F3 A-2.10	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCATTGG CCA TCT ACA AGC AGT CA	200N
Exon 5 F3 P-2.1	CCT CTC TAT GGG CAG TCG GTG ATAGGT GGC CAT CTA CAA GCA GTC A	200N
Exon 5 F3 P-2.2	CCT CTC TAT GGG CAG TCG GTG ATAGTT GGC CAT CTA CAA GCA GTC A	200N
Exon 5 F3 P-2.3	CCT CTC TAT GGG CAG TCG GTG ATATAT GGC CAT CTA CAA GCA GTC A	200N
Exon 5 F3 P-2.4	CCT CTC TAT GGG CAG TCG GTG ATATCT GGC CAT CTA CAA GCA GTC A	200N
Exon 5 F3 P-2.5	CCT CTC TAT GGG CAG TCG GTG ATATGT GGC CAT CTA CAA GCA GTC A	200N
Exon 5 F3 P-2.6	CCT CTC TAT GGG CAG TCG GTG ATATTT GGC CAT CTA CAA GCA GTC A	200N
Exon 5 F3 P-2.7	CCT CTC TAT GGG CAG TCG GTG ATCAAT GGC CAT CTA CAA GCA GTC A	200N
Exon 5 F3 P-2.8	CCT CTC TAT GGG CAG TCG GTG ATCACT GGC CAT CTA CAA GCA GTC A	200N
Exon 5 F3 P-2.9	CCT CTC TAT GGG CAG TCG GTG ATCAGT GGC CAT CTA CAA GCA GTC A	200N
Exon 5 F3 P-2.10	CCT CTC TAT GGG CAG TCG GTG ATCATT GGC CAT CTA CAA GCA GTC A	200N
Exon 6 F A-2.1	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGGCAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F A-2.2	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGTCAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F A-2.3	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATACAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F A-2.4	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATCCAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F A-2.5	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATGCAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F A-2.6	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATTCAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F A-2.7	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCAACAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F A-2.8	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCACCAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F A-2.9	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCAGCAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F A-2.10	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCATCAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F P-2.1	CCT CTC TAT GGG CAG TCG GTG ATAGGC AGG CCT CTG ATT CCT CAC T	200N
Exon 6 F P-2.2	CCT CTC TAT GGG CAG TCG GTG ATAGTC AGG CCT CTG ATT CCT CAC T	200N
Exon 6 F P-2.3	CCT CTC TAT GGG CAG TCG GTG ATATAC AGG CCT CTG ATT CCT CAC T	200N
Exon 6 F P-2.4	CCT CTC TAT GGG CAG TCG GTG ATATCC AGG CCT CTG ATT CCT CAC T	200N
Exon 6 F P-2.5	CCT CTC TAT GGG CAG TCG GTG ATATGC AGG CCT CTG ATT CCT CAC T	200N
Exon 6 F P-2.6	CCT CTC TAT GGG CAG TCG GTG ATATTC AGG CCT CTG ATT CCT CAC T	200N
Exon 6 F P-2.7	CCT CTC TAT GGG CAG TCG GTG ATCAAC AGG CCT CTG ATT CCT CAC T	200N
Exon 6 F P-2.8	CCT CTC TAT GGG CAG TCG GTG ATCACC AGG CCT CTG ATT CCT CAC T	200N
Exon 6 F P-2.9	CCT CTC TAT GGG CAG TCG GTG ATCAGC AGG CCT CTG ATT CCT CAC T	200N
Exon 6 F P-2.10	CCT CTC TAT GGG CAG TCG GTG ATCATC AGG CCT CTG ATT CCT CAC T	200N
Exon 7 F1 A-2.1	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGGCCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 A-2.2	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGTCCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 A-2.3	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATACCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 A-2.4	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATCCCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 A-2.5	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATGCCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 A-2.6	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATTCCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 A-2.7	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCAACCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 A-2.8	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCACCCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 A-2.9	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCAGCCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 A-2.10	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCATCCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 P-2.1	CCT CTC TAT GGG CAG TCG GTG ATAGGC CTG CTT GCC ACA GGT CT	200N
Exon 7 F1 P-2.2	CCT CTC TAT GGG CAG TCG GTG ATAGTC CTG CTT GCC ACA GGT CT	200N
Exon 7 F1 P-2.3	CCT CTC TAT GGG CAG TCG GTG ATATAC CTG CTT GCC ACA GGT CT	200N

Exon 7 F1 P-2.4	CCT CTC TAT GGG CAG TCG GTG ATATCC CTG CTT GCC ACA GGT CT	200N
Exon 7 F1 P-2.5	CCT CTC TAT GGG CAG TCG GTG ATATGC CTG CTT GCC ACA GGT CT	200N
Exon 7 F1 P-2.6	CCT CTC TAT GGG CAG TCG GTG ATATTC CTG CTT GCC ACA GGT CT	200N
Exon 7 F1 P-2.7	CCT CTC TAT GGG CAG TCG GTG ATCAAC CTG CTT GCC ACA GGT CT	200N
Exon 7 F1 P-2.8	CCT CTC TAT GGG CAG TCG GTG ATCACC CTG CTT GCC ACA GGT CT	200N
Exon 7 F1 P-2.9	CCT CTC TAT GGG CAG TCG GTG ATCAGC CTG CTT GCC ACA GGT CT	200N
Exon 7 F1 P-2.10	CCT CTC TAT GGG CAG TCG GTG ATCATC CTG CTT GCC ACA GGT CT	200N
Exon 7 F2 A-2.1	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGGCCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 A-2.2	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGTCCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 A-2.3	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATACCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 A-2.4	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATCCCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 A-2.5	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATGCCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 A-2.6	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATTCCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 A-2.7	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCAACCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 A-2.8	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCACCCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 A-2.9	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCAGCCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 A-2.10	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCATCCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 P-2.1	CCT CTC TAT GGG CAG TCG GTG ATAGGC CTC ACC ATC ATC ACA CTG G	200N
Exon 7 F2 P-2.2	CCT CTC TAT GGG CAG TCG GTG ATAGTC CTC ACC ATC ATC ACA CTG G	200N
Exon 7 F2 P-2.3	CCT CTC TAT GGG CAG TCG GTG ATATAC CTC ACC ATC ATC ACA CTG G	200N
Exon 7 F2 P-2.4	CCT CTC TAT GGG CAG TCG GTG ATATCC CTC ACC ATC ATC ACA CTG G	200N
Exon 7 F2 P-2.5	CCT CTC TAT GGG CAG TCG GTG ATATGC CTC ACC ATC ATC ACA CTG G	200N
Exon 7 F2 P-2.6	CCT CTC TAT GGG CAG TCG GTG ATATTC CTC ACC ATC ATC ACA CTG G	200N
Exon 7 F2 P-2.7	CCT CTC TAT GGG CAG TCG GTG ATCAAC CTC ACC ATC ATC ACA CTG G	200N
Exon 7 F2 P-2.8	CCT CTC TAT GGG CAG TCG GTG ATCACC CTC ACC ATC ATC ACA CTG G	200N
Exon 7 F2 P-2.9	CCT CTC TAT GGG CAG TCG GTG ATCAGC CTC ACC ATC ATC ACA CTG G	200N
Exon 7 F2 P-2.10	CCT CTC TAT GGG CAG TCG GTG ATCATC CTC ACC ATC ATC ACA CTG G	200N
Exon 8 F1 A-2.1	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGGTGA TTT CCT TAC TGC CTC TTGC	200N
Exon 8 F1 A-2.2	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGTTGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 A-2.3	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATATGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 A-2.4	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATCTGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 A-2.5	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATGTGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 A-2.6	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATTTGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 A-2.7	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCAATGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 A-2.8	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCACTGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 A-2.9	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCAGTGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 A-2.10	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCATTGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 P-2.1	CCT CTC TAT GGG CAG TCG GTG ATAGGT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F1 P-2.2	CCT CTC TAT GGG CAG TCG GTG ATAGTT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F1 P-2.3	CCT CTC TAT GGG CAG TCG GTG ATATAT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F1 P-2.4	CCT CTC TAT GGG CAG TCG GTG ATATCT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F1 P-2.5	CCT CTC TAT GGG CAG TCG GTG ATATGT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F1 P-2.6	CCT CTC TAT GGG CAG TCG GTG ATATTT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F1 P-2.7	CCT CTC TAT GGG CAG TCG GTG ATCAAT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F1 P-2.8	CCT CTC TAT GGG CAG TCG GTG ATCACT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F1 P-2.9	CCT CTC TAT GGG CAG TCG GTG ATCAGT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F1 P-2.10	CCT CTC TAT GGG CAG TCG GTG ATCATT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F2 A-2.1	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGGACT GGG ACG GAA CAG CTT TG	200N
Exon 8 F2 A-2.2	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGAGTACT GGG ACG GAA CAG CTT TG	200N
Exon 8 F2 A-2.3	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATAACT GGG ACG GAA CAG CTT TG	200N
Exon 8 F2 A-2.4	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATCACT GGG ACG GAA CAG CTT TG	200N
Exon 8 F2 A-2.5	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATGACT GGG ACG GAA CAG CTT TG	200N

Exon 8 F2 A-2.6	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGATTACT GGG ACG GAA CAG CTT TG	200N
Exon 8 F2 A-2.7	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCAAACT GGG ACG GAA CAG CTT TG	200N
Exon 8 F2 A-2.8	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCACACT GGG ACG GAA CAG CTT TG	200N
Exon 8 F2 A-2.9	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCAGACT GGG ACG GAA CAG CTT TG	200N
Exon 8 F2 A-2.10	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCATACT GGG ACG GAA CAG CTT TG	200N
Exon 8 F2 P-2.1	CCT CTC TAT GGG CAG TCG GTG ATAGGA CTG GGA CGG AAC AGC TTT G	200N
Exon 8 F2 P-2.2	CCT CTC TAT GGG CAG TCG GTG ATAGTA CTG GGA CGG AAC AGC TTT G	200N
Exon 8 F2 P-2.3	CCT CTC TAT GGG CAG TCG GTG ATATAA CTG GGA CGG AAC AGC TTT G	200N
Exon 8 F2 P-2.4	CCT CTC TAT GGG CAG TCG GTG ATATCA CTG GGA CGG AAC AGC TTT G	200N
Exon 8 F2 P-2.5	CCT CTC TAT GGG CAG TCG GTG ATATGA CTG GGA CGG AAC AGC TTT G	200N
Exon 8 F2 P-2.6	CCT CTC TAT GGG CAG TCG GTG ATATTA CTG GGA CGG AAC AGC TTT G	200N
Exon 8 F2 P-2.7	CCT CTC TAT GGG CAG TCG GTG ATCAAA CTG GGA CGG AAC AGC TTT G	200N
Exon 8 F2 P-2.8	CCT CTC TAT GGG CAG TCG GTG ATCACA CTG GGA CGG AAC AGC TTT G	200N
Exon 8 F2 P-2.9	CCT CTC TAT GGG CAG TCG GTG ATCAGA CTG GGA CGG AAC AGC TTT G	200N
Exon 8 F2 P-2.10	CCT CTC TAT GGG CAG TCG GTG ATCATA CTG GGA CGG AAC AGC TTT G	200N
Exon 5 F1 A-3.1	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCACAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 A-3.2	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCCCAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 A-3.3	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCGCAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 A-3.4	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCTCAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 A-3.5	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGACAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 A-3.6	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGCCAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 A-3.7	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGGCAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 A-3.8	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGTCAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 A-3.9	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCTACAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 A-3.10	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCTCCAC TTG TGC CCT GAC TTT CA	200N
Exon 5 F1 P-3.1	CCT CTC TAT GGG CAG TCG GTG ATCCAC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F1 P-3.2	CCT CTC TAT GGG CAG TCG GTG ATCCCC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F1 P-3.3	CCT CTC TAT GGG CAG TCG GTG ATCCGC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F1 P-3.4	CCT CTC TAT GGG CAG TCG GTG ATCCTC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F1 P-3.5	CCT CTC TAT GGG CAG TCG GTG ATCGAC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F1 P-3.6	CCT CTC TAT GGG CAG TCG GTG ATCGCC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F1 P-3.7	CCT CTC TAT GGG CAG TCG GTG ATCGGC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F1 P-3.8	CCT CTC TAT GGG CAG TCG GTG ATCGTC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F1 P-3.9	CCT CTC TAT GGG CAG TCG GTG ATCTAC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F1 P-3.10	CCT CTC TAT GGG CAG TCG GTG ATCTCC ACT TGT GCC CTG ACT TTC A	200N
Exon 5 F2 A-3.1	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCAAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 A-3.2	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCCAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 A-3.3	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCGAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 A-3.4	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCTAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 A-3.5	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGAAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 A-3.6	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGCAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 A-3.7	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGGAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 A-3.8	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGTAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 A-3.9	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCTAAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 A-3.10	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCTCAGT ACT CCC CTG CCC TCA AC	200N
Exon 5 F2 P-3.1	CCT CTC TAT GGG CAG TCG GTG ATCCAA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F2 P-3.2	CCT CTC TAT GGG CAG TCG GTG ATCCCA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F2 P-3.3	CCT CTC TAT GGG CAG TCG GTG ATCCGA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F2 P-3.4	CCT CTC TAT GGG CAG TCG GTG ATCCTA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F2 P-3.5	CCT CTC TAT GGG CAG TCG GTG ATCGAA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F2 P-3.6	CCT CTC TAT GGG CAG TCG GTG ATCGCA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F2 P-3.7	CCT CTC TAT GGG CAG TCG GTG ATCGGA GTA CTC CCC TGC CCT CAA C	200N

Exon 5 F2 P-3.8	CCT CTC TAT GGG CAG TCG GTG ATCGTA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F2 P-3.9	CCT CTC TAT GGG CAG TCG GTG ATCTAA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F2 P-3.10	CCT CTC TAT GGG CAG TCG GTG ATCTCA GTA CTC CCC TGC CCT CAA C	200N
Exon 5 F3 A-3.1	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCATGG CCA TCT ACA AGC AGT CA	200N
Exon 5 F3 A-3.2	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCCTGG CCA TCT ACA AGC AGT CA	200N
Exon 5 F3 A-3.3	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCGTGG CCA TCT ACA AGC AGT CA	200N
Exon 5 F3 A-3.4	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCTTGG CCA TCT ACA AGC AGT CA	200N
Exon 5 F3 A-3.5	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGATGG CCA TCT ACA AGC AGT CA	200N
Exon 5 F3 A-3.6	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGCTGG CCA TCT ACA AGC AGT CA	200N
Exon 5 F3 A-3.7	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGGTGG CCA TCT ACA AGC AGT CA	200N
Exon 5 F3 A-3.8	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGTTGG CCA TCT ACA AGC AGT CA	200N
Exon 5 F3 A-3.9	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCTATGG CCA TCT ACA AGC AGT CA	200N
Exon 5 F3 A-3.10	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCTCTGG CCA TCT ACA AGC AGT CA	200N
Exon 5 F3 P-3.1	CCT CTC TAT GGG CAG TCG GTG ATCCAT GGC CAT CTA CAA GCA GTC A	200N
Exon 5 F3 P-3.2	CCT CTC TAT GGG CAG TCG GTG ATCCCT GGC CAT CTA CAA GCA GTC A	200N
Exon 5 F3 P-3.3	CCT CTC TAT GGG CAG TCG GTG ATCCGT GGC CAT CTA CAA GCA GTC A	200N
Exon 5 F3 P-3.4	CCT CTC TAT GGG CAG TCG GTG ATCCTT GGC CAT CTA CAA GCA GTC A	200N
Exon 5 F3 P-3.5	CCT CTC TAT GGG CAG TCG GTG ATCGAT GGC CAT CTA CAA GCA GTC A	200N
Exon 5 F3 P-3.6	CCT CTC TAT GGG CAG TCG GTG ATCGCT GGC CAT CTA CAA GCA GTC A	200N
Exon 5 F3 P-3.7	CCT CTC TAT GGG CAG TCG GTG ATCGGT GGC CAT CTA CAA GCA GTC A	200N
Exon 5 F3 P-3.8	CCT CTC TAT GGG CAG TCG GTG ATCGTT GGC CAT CTA CAA GCA GTC A	200N
Exon 5 F3 P-3.9	CCT CTC TAT GGG CAG TCG GTG ATCTAT GGC CAT CTA CAA GCA GTC A	200N
Exon 5 F3 P-3.10	CCT CTC TAT GGG CAG TCG GTG ATCTCT GGC CAT CTA CAA GCA GTC A	200N
Exon 6 F A-3.1	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCACAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F A-3.2	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCCCAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F A-3.3	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCGCAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F A-3.4	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCTCAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F A-3.5	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGACAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F A-3.6	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGCCAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F A-3.7	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGGCAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F A-3.8	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGTCAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F A-3.9	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCTACAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F A-3.10	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCTCCAG GCC TCT GAT TCC TCA CT	200N
Exon 6 F P-3.1	CCT CTC TAT GGG CAG TCG GTG ATCCAC AGG CCT CTG ATT CCT CAC T	200N
Exon 6 F P-3.2	CCT CTC TAT GGG CAG TCG GTG ATCCCC AGG CCT CTG ATT CCT CAC T	200N
Exon 6 F P-3.3	CCT CTC TAT GGG CAG TCG GTG ATCCGC AGG CCT CTG ATT CCT CAC T	200N
Exon 6 F P-3.4	CCT CTC TAT GGG CAG TCG GTG ATCCTC AGG CCT CTG ATT CCT CAC T	200N
Exon 6 F P-3.5	CCT CTC TAT GGG CAG TCG GTG ATCGAC AGG CCT CTG ATT CCT CAC T	200N
Exon 6 F P-3.6	CCT CTC TAT GGG CAG TCG GTG ATCGCC AGG CCT CTG ATT CCT CAC T	200N
Exon 6 F P-3.7	CCT CTC TAT GGG CAG TCG GTG ATCGGC AGG CCT CTG ATT CCT CAC T	200N
Exon 6 F P-3.8	CCT CTC TAT GGG CAG TCG GTG ATCGTC AGG CCT CTG ATT CCT CAC T	200N
Exon 6 F P-3.9	CCT CTC TAT GGG CAG TCG GTG ATCTAC AGG CCT CTG ATT CCT CAC T	200N
Exon 6 F P-3.10	CCT CTC TAT GGG CAG TCG GTG ATCTCC AGG CCT CTG ATT CCT CAC T	200N
Exon 7 F1 A-3.1	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCACCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 A-3.2	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCCCCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 A-3.3	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCGCCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 A-3.4	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCTCCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 A-3.5	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGACCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 A-3.6	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGCCCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 A-3.7	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGGCCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 A-3.8	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGTCCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 A-3.9	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCTACCT GCT TGC CAC AGG TCT	200N

Exon 7 F1 A-3.10	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCTCCCT GCT TGC CAC AGG TCT	200N
Exon 7 F1 P-3.1	CCT CTC TAT GGG CAG TCG GTG ATCCAC CTG CTT GCC ACA GGT CT	200N
Exon 7 F1 P-3.2	CCT CTC TAT GGG CAG TCG GTG ATCCCC CTG CTT GCC ACA GGT CT	200N
Exon 7 F1 P-3.3	CCT CTC TAT GGG CAG TCG GTG ATCCGC CTG CTT GCC ACA GGT CT	200N
Exon 7 F1 P-3.4	CCT CTC TAT GGG CAG TCG GTG ATCCTC CTG CTT GCC ACA GGT CT	200N
Exon 7 F1 P-3.5	CCT CTC TAT GGG CAG TCG GTG ATCGAC CTG CTT GCC ACA GGT CT	200N
Exon 7 F1 P-3.6	CCT CTC TAT GGG CAG TCG GTG ATCGCC CTG CTT GCC ACA GGT CT	200N
Exon 7 F1 P-3.7	CCT CTC TAT GGG CAG TCG GTG ATCGGC CTG CTT GCC ACA GGT CT	200N
Exon 7 F1 P-3.8	CCT CTC TAT GGG CAG TCG GTG ATCGTC CTG CTT GCC ACA GGT CT	200N
Exon 7 F1 P-3.9	CCT CTC TAT GGG CAG TCG GTG ATCTAC CTG CTT GCC ACA GGT CT	200N
Exon 7 F1 P-3.10	CCT CTC TAT GGG CAG TCG GTG ATCTCC CTG CTT GCC ACA GGT CT	200N
Exon 7 F2 A-3.1	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCACCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 A-3.2	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCCCCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 A-3.3	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCGCCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 A-3.4	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCTCCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 A-3.5	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGACCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 A-3.6	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGCCCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 A-3.7	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGGCCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 A-3.8	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGTCCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 A-3.9	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCTACCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 A-3.10	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCTCCCT CAC CAT CAT CAC ACT GG	200N
Exon 7 F2 P-3.1	CCT CTC TAT GGG CAG TCG GTG ATCCAC CTC ACC ATC ATC ACA CTG G	200N
Exon 7 F2 P-3.2	CCT CTC TAT GGG CAG TCG GTG ATCCCC CTC ACC ATC ATC ACA CTG G	200N
Exon 7 F2 P-3.3	CCT CTC TAT GGG CAG TCG GTG ATCCGC CTC ACC ATC ATC ACA CTG G	200N
Exon 7 F2 P-3.4	CCT CTC TAT GGG CAG TCG GTG ATCCTC CTC ACC ATC ATC ACA CTG G	200N
Exon 7 F2 P-3.5	CCT CTC TAT GGG CAG TCG GTG ATCGAC CTC ACC ATC ATC ACA CTG G	200N
Exon 7 F2 P-3.6	CCT CTC TAT GGG CAG TCG GTG ATCGCC CTC ACC ATC ATC ACA CTG G	200N
Exon 7 F2 P-3.7	CCT CTC TAT GGG CAG TCG GTG ATCGGC CTC ACC ATC ATC ACA CTG G	200N
Exon 7 F2 P-3.8	CCT CTC TAT GGG CAG TCG GTG ATCGTC CTC ACC ATC ATC ACA CTG G	200N
Exon 7 F2 P-3.9	CCT CTC TAT GGG CAG TCG GTG ATCTAC CTC ACC ATC ATC ACA CTG G	200N
Exon 7 F2 P-3.10	CCT CTC TAT GGG CAG TCG GTG ATCTCC CTC ACC ATC ATC ACA CTG G	200N
Exon 8 F1 A-3.1	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCATGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 A-3.2	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCCTGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 A-3.3	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCGTGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 A-3.4	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCTTGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 A-3.5	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGATGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 A-3.6	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGCTGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 A-3.7	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGGTGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 A-3.8	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGTTGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 A-3.9	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCTATGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 A-3.10	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCTCTGA TTT CCT TAC TGC CTC TTG C	200N
Exon 8 F1 P-3.1	CCT CTC TAT GGG CAG TCG GTG ATCCAT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F1 P-3.2	CCT CTC TAT GGG CAG TCG GTG ATCCCT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F1 P-3.3	CCT CTC TAT GGG CAG TCG GTG ATCCGT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F1 P-3.4	CCT CTC TAT GGG CAG TCG GTG ATCCTT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F1 P-3.5	CCT CTC TAT GGG CAG TCG GTG ATCGAT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F1 P-3.6	CCT CTC TAT GGG CAG TCG GTG ATCGCT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F1 P-3.7	CCT CTC TAT GGG CAG TCG GTG ATCGGT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F1 P-3.8	CCT CTC TAT GGG CAG TCG GTG ATCGTT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F1 P-3.9	CCT CTC TAT GGG CAG TCG GTG ATCTAT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F1 P-3.10	CCT CTC TAT GGG CAG TCG GTG ATCTCT GAT TTC CTT ACT GCC TCT TGC	200N
Exon 8 F2 A-3.1	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCAACT GGG ACG GAA CAG CTT TG	200N

Exon 8 F2 A-3.2	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCCACT GGG ACG GAA CAG CTT TG	200N
Exon 8 F2 A-3.3	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCGACT GGG ACG GAA CAG CTT TG	200N
Exon 8 F2 A-3.4	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCCTACT GGG ACG GAA CAG CTT TG	200N
Exon 8 F2 A-3.5	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGAACT GGG ACG GAA CAG CTT TG	200N
Exon 8 F2 A-3.6	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGCACT GGG ACG GAA CAG CTT TG	200N
Exon 8 F2 A-3.7	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGGACT GGG ACG GAA CAG CTT TG	200N
Exon 8 F2 A-3.8	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCGTACT GGG ACG GAA CAG CTT TG	200N
Exon 8 F2 A-3.9	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCTAACT GGG ACG GAA CAG CTT TG	200N
Exon 8 F2 A-3.10	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAGCTCACT GGG ACG GAA CAG CTT TG	200N
Exon 8 F2 P-3.1	CCT CTC TAT GGG CAG TCG GTG ATCCAA CTG GGA CGG AAC AGC TTT G	200N
Exon 8 F2 P-3.2	CCT CTC TAT GGG CAG TCG GTG ATCCCA CTG GGA CGG AAC AGC TTT G	200N
Exon 8 F2 P-3.3	CCT CTC TAT GGG CAG TCG GTG ATCCGA CTG GGA CGG AAC AGC TTT G	200N
Exon 8 F2 P-3.4	CCT CTC TAT GGG CAG TCG GTG ATCCTA CTG GGA CGG AAC AGC TTT G	200N
Exon 8 F2 P-3.5	CCT CTC TAT GGG CAG TCG GTG ATCGAA CTG GGA CGG AAC AGC TTT G	200N
Exon 8 F2 P-3.6	CCT CTC TAT GGG CAG TCG GTG ATCGCA CTG GGA CGG AAC AGC TTT G	200N
Exon 8 F2 P-3.7	CCT CTC TAT GGG CAG TCG GTG ATCGGA CTG GGA CGG AAC AGC TTT G	200N
Exon 8 F2 P-3.8	CCT CTC TAT GGG CAG TCG GTG ATCGTA CTG GGA CGG AAC AGC TTT G	200N
Exon 8 F2 P-3.9	CCT CTC TAT GGG CAG TCG GTG ATCTAA CTG GGA CGG AAC AGC TTT G	200N
Exon 8 F2 P-3.10	CCT CTC TAT GGG CAG TCG GTG ATCTCA CTG GGA CGG AAC AGC TTT G	200N
Exon5 R1A	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG	200N
Exon5 R1P	CCT CTC TAT GGG CAG TCG GTG AT	200N
Exon5R2A	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG	200N
Exon5 R2P	CCT CTC TAT GGG CAG TCG GTG AT	200N
ExoN5 R3A	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG	200N
Exon5 R3P	CCT CTC TAT GGG CAG TCG GTG AT	200N
Exon6 RA	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG	200N
Exon6 RP	CCT CTC TAT GGG CAG TCG GTG AT	200N
Exon7 R1A	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG	200N
Exon7 R1P	CCT CTC TAT GGG CAG TCG GTG AT	200N
Exon7 R2A	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG	200N
Exon7 R2P	CCT CTC TAT GGG CAG TCG GTG AT	200N
Exon8 R1A	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG	200N
Exon8 R1P	CCT CTC TAT GGG CAG TCG GTG AT	200N
Exon8 R2A	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG	200N
Exon8 R2P	CCT CTC TAT GGG CAG TCG GTG AT	200N

Appendix D: World Health Organisation TNM staging for pancreatic cancer.

Primary tumour (T)	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ
T1	Tumour limited to the pancreas, ≤ 2 cm in greatest dimension
T2	Tumour limited to the pancreas, > 2 cm in greatest dimension
T3	Tumour extends beyond the pancreas but without involvement of the celiac axis or the superior mesenteric artery
T4	Tumour involves the celiac axis or the superior mesenteric artery (unresectable primary tumour)
Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis
Distant metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis

[Guideline] National Comprehensive Cancer Network. NCCN Clinical Practice Guidelines in Oncology: Pancreatic Adenocarcinoma. V 2.2015. Available at http://www.nccn.org/professionals/physician_gls/pdf/pancreatic.pdf. Accessed: December 30, 2015.

American Joint Committee on Cancer (AJCC) TNM staging system, September 6, 2013. American Cancer Society. Available at http://www.cancer.org/cancer/pancreaticcancer/detailedguide/pancreatic-cancer-staging. Accessed: December 30, 2015.

Appendix E: List of read depths for all chips used on the Ion Torrent.

CHIP	CYST -	Read	Average Fragment Length	Average Depth of Reads for Each Fragment (Exon, Fragment)							
				E5F1	E5F2	E5F3	E6	E7F1	E72	E81	E82
1	1	1	78 bp	2120	1399	10801	82	7913	8306	4795	653
		2	71 bp	3249	6836	12146	198	347	7638	2863	619
		3	68 bp	5827	2859	5038	82	4129	8559	4822	75
		4	72 bp	7259	6520	4008	404	7919	9110	2831	239
		5	48 bp	2243	1643	7548	388	5851	491	5270	693
		6	51 bp	4594	584	11392	441	5777	5497	361	569
		7	70 bp	556	7593	7194	165	4344	8814	4000	768
		8	51 bp	2913	544	8673	241	1287	3423	1615	511
		9	52 bp	9559	3795	3481	310	749	1987	2433	570
		10	54 bp	10370	9434	7243	379	5697	8816	3806	140
	2	1	83 bp	2421	9284	10365	31	2029	4640	4754	230
		2	48 bp	3364	2094	1136	462	3760	697	5535	319
		3	63 bp	187	126	4638	22	3183	7131	4210	378
		4	47 bp	6353	824	539	458	7882	7679	4451	511
		5	57 bp	7089	3827	4762	123	2331	4630	2673	551
		6	54 bp	5544	2588	3640	123	7264	4653	5221	556
		7	63 bp	4997	8026	7511	373	1695	4336	3574	696
		8	42 bp	8268	9581	4417	283	5131	866	3630	953
		9	44 bp	5289	9711	5950	287	6237	7504	204	827
		10	43 bp	5711	6693	1275	331	3254	1137	2037	273
	3	1	86 bp	5579	3192	9003	165	5473	1272	731	686
		2	49 bp	2984	2873	4959	245	7794	165	2873	206
		3	66 bp	9045	4262	2146	56	22	8100	3155	314
		4	47 bp	8704	4371	6863	120	1852	5621	2873	119
		5	61 bp	1603	3125	10301	40	7042	5572	3390	884
		6	62 bp	7722	1064	12160	388	7191	4622	2643	897
		7	84 bp	6947	7778	840	406	2826	4634	872	436
		8	68 bp	6798	1956	8796	169	6523	4803	333	276
		9	50 bp	1132	9836	8217	160	4670	2151	3346	672
		10	62 bp	2239	6276	6669	382	4731	6775	5120	764
2	4	1	63 bp	11469	6756	3938	301	1208	9044	3140	525
		2	51 bp	2284	1107	7634	188	4909	5607	3482	758
		3	53 bp	7203	1722	621	88	3687	7808	3250	77
		4	48 bp	2591	6201	921	45	6711	4706	2007	614
		5	49 bp	10240	8030	2119	440	772	3818	1321	654
		6	42 bp	10453	693	7905	179	4222	3710	3970	850
		7	51 bp	4855	929	7558	307	6411	3905	2177	75
		8	43 bp	9097	4202	3929	459	2170	7847	5949	140
		9	44 bp	8280	9630	4987	386	1275	5913	3358	409

		10	55 bp	2973	2948	10063	438	4772	8156	4829	418
	5	1	0 bp	0	0	0	0	0	0	0	0
		2	0 bp	0	0	0	0	0	0	0	0
		3	0 bp	0	0	0	0	0	0	0	0
		4	0 bp	0	0	0	0	0	0	0	0
		5	0 bp	0	0	0	0	0	0	0	0
		6	0 bp	0	0	0	0	0	0	0	0
		7	0 bp	0	0	0	0	0	0	0	0
		8	0 bp	0	0	0	0	0	0	0	0
		9	0 bp	0	0	0	0	0	0	0	0
		10	0 bp	0	0	0	0	0	0	0	0
	6	1	56 bp	2217	5758	2088	456	4557	7990	1812	740
		2	57 bp	174	8321	9231	223	2001	7978	435	949
		3	58 bp	8921	3516	8417	268	7399	1242	762	349
		4	50 bp	10850	8472	8551	345	6576	3216	2881	360
		5	78 bp	9130	2321	3502	162	7425	4410	211	146
		6	58 bp	9027	1841	2009	397	6391	7461	3579	247
		7	77 bp	2916	1625	1785	3	7840	9556	3246	862
		8	44 bp	11080	2163	10361	487	7362	1534	551	318
		9	33 bp	3089	1740	8227	456	7465	6430	4634	203
		10	58 bp	10961	3593	6059	286	1248	5466	1876	743
3	7	1	75 bp	2948	3200	7842	440	6529	9459	4554	495
		2	56 bp	9632	3742	7640	294	7162	9036	4106	950
		3	68 bp	10630	9662	2195	484	6658	4500	2264	292
		4	65 bp	10367	1346	10791	241	7328	3647	2599	50
		5	55 bp	7356	7735	1804	290	1055	4128	2173	781
		6	60 bp	4911	947	7623	480	3065	6118	1463	322
		7	73 bp	453	3661	9825	434	7128	8887	5903	411
		8	43 bp	10591	8414	7310	310	7738	512	1678	66
		9	72 bp	8988	9244	2012	425	7619	8127	4703	486
		10	74 bp	2198	7899	7387	334	7967	8203	5668	892
	8	1	86 bp	6105	8369	2899	320	1895	327	62	613
		2	52 bp	11360	3225	10569	299	3647	8178	5171	365
		3	73 bp	9299	8765	11479	365	1822	5329	4029	781
		4	77 bp	4297	9726	902	97	4415	2639	2072	328
		5	65 bp	9278	1061	2799	149	5405	6901	4467	90
		6	69 bp	8546	1013	4910	356	6987	2614	1068	979
		7	63 bp	7757	6338	8963	81	6897	1459	5427	351
		8	67 bp	10804	76	2802	136	3968	7454	1097	830
		9	70 bp	10366	9461	8726	421	6516	6733	3550	457
		10	88 bp	11680	4375	9502	25	7171	6603	1315	177
	9	1	68 bp	6170	4821	10781	168	6373	8224	5365	725
		2	62 bp	9012	377	11372	389	3701	7920	153	870
		3	66 bp	8339	3692	7116	209	6228	8444	2367	218

		4	72 bp	3688	12	5452	124	6983	9326	4569	286
		5	80 bp	7813	921	3013	17	621	2285	328	187
		6	60 bp	5566	3712	8252	395	2611	934	2256	243
		7	81 bp	2340	1545	4279	408	6891	6239	4563	319
		8	55 bp	5135	3632	7626	72	7464	6284	4107	813
		9	55 bp	4673	4510	9946	289	7235	8076	3879	560
		10	68 bp	807	9627	8260	211	6911	1214	659	755
4	10	1	86 bp	2393	6584	11706	202	1315	2898	4213	56
		2	96 bp	11605	9014	1992	211	2795	8484	3923	139
		3	95 bp	4747	1385	12161	441	7016	1119	5731	492
		4	80 bp	5944	6704	4062	371	1947	1738	5235	979
		5	79 bp	11693	7023	5906	230	2607	3305	5759	567
		6	69 bp	8913	6681	1846	294	3673	544	1081	951
		7	84 bp	4166	6301	9578	375	4503	6194	568	265
		8	106 bp	5903	7518	9079	391	2021	3998	1630	82
		9	84 bp	9747	8402	6659	285	6008	5308	5114	448
		10	100 bp	10002	5188	8543	425	1377	1108	473	228
	11	1	106 bp	217	7590	7907	452	6247	1575	4357	901
		2	78 bp	4823	8710	10161	330	2413	9027	3443	292
		3	93 bp	8062	8426	256	189	1579	2481	4929	725
		4	91 bp	10172	2955	12164	162	4721	9166	3091	434
		5	85 bp	4991	1231	10615	153	4005	7268	5137	741
		6	91 bp	4278	9234	10548	355	4861	2499	204	321
		7	82 bp	4720	6916	1727	315	3211	5821	2819	695
		8	74 bp	3958	1636	5528	463	134	868	4376	187
		9	44 bp	4261	6415	10127	21	2632	7664	5595	790
		10	45 bp	120	2624	4552	415	7292	8319	2020	585
	12	1	94 bp	3858	2484	3889	402	6279	8122	58	581
		2	87 bp	656	2627	9730	286	2995	7227	339	355
		3	90 bp	2382	2132	1478	121	2118	3980	5700	676
		4	88 bp	287	7791	12282	208	3957	6055	1138	502
		5	75 bp	3357	2836	10903	145	6807	1839	4486	645
		6	86 bp	1337	8766	2100	231	1184	6697	2749	219
		7	88 bp	9885	8865	1935	362	2256	3857	5314	412
		8	71 bp	3062	9344	1081	348	2848	5842	3128	247
		9	77 bp	11544	5008	1885	30	7502	3720	959	952
		10	81 bp	9398	1390	3222	449	2087	9166	4191	904
5	13	1	89 bp	6116	4227	1867	17	2838	435	1286	773
		2	86 bp	1708	4257	4607	285	3252	9234	2441	822
		3	117 bp	2844	1763	9201	64	1776	5995	2892	613
		4	80 bp	11191	6022	10412	434	5330	9235	3642	665
		5	66 bp	5404	8176	5579	453	283	8445	1771	467
		6	109 bp	9484	6141	8900	70	213	4947	2739	176
		7	96 bp	6538	3621	12033	409	3756	7651	2697	920

		8	87 bp	4113	7514	8547	131	7132	1802	5229	528
		9	99 bp	8887	2010	12547	52	4233	7643	3615	211
		10	104 bp	11382	3473	9796	341	5513	7356	555	278
	14	1	93 bp	6556	4835	973	175	5572	1857	1287	115
		2	47 bp	11263	1503	4747	37	7813	6710	3193	245
		3	61 bp	3897	409	8997	266	2018	2999	1961	339
		4	68 bp	6697	5818	11923	164	5959	3142	2647	457
		5	63 bp	5224	9541	10108	426	1631	1500	1012	333
		6	62 bp	2894	6349	4945	308	6888	1664	1177	802
		7	104 bp	4617	1285	12314	21	3292	259	5895	193
		8	106 bp	7900	5720	8042	66	7565	6810	1940	527
		9	51 bp	1342	753	7795	338	2223	8051	845	955
		10	50 bp	7519	979	11537	151	2016	5338	4818	242
	15	1	73 bp	5118	8612	10694	424	1116	3113	4707	399
		2	41 bp	4487	8165	5335	205	6753	6787	2705	498
		3	111 bp	1978	6979	1510	107	3421	9199	921	868
		4	80 bp	9825	5839	6094	474	456	8911	1593	279
		5	137 bp	5168	7147	11197	37	4216	1017	3863	765
		6	98 bp	1809	6672	10811	121	498	7337	2019	680
		7	100 bp	69	4901	356	172	7140	8852	3204	57
		8	86 bp	4143	5838	1016	282	2891	9020	648	612
		9	76 bp	11451	4531	11629	266	3445	3374	1487	172
		10	86 bp	6106	4888	11196	16	2919	2849	11	433
6	16	1	47 bp	9704	5087	8808	445	775	7440	4684	308
		2	46 bp	11143	7799	8172	467	712	2185	4240	460
		3	41 bp	10275	1750	3174	71	5161	7650	2023	641
		4	42 bp	10288	2183	5678	68	367	9356	4319	156
		5	39 bp	10922	9584	578	294	7153	763	267	262
		6	39 bp	2418	8032	9188	224	2910	6564	2272	506
		7	44 bp	2099	9711	1559	378	3578	8630	1133	125
		8	33 bp	9142	6652	8303	105	3375	8536	4300	935
		9	36 bp	1834	8025	10708	152	710	80	82	758
		10	46 bp	2449	8189	7630	257	4067	3588	4681	246
	17	1	50 bp	9665	8262	2388	360	4583	3964	2346	363
		2	43 bp	5844	9250	6808	254	1579	3623	5529	467
		3	42 bp	8475	2553	10391	157	7392	9484	2982	249
		4	46 bp	5368	5523	10145	39	952	1398	1611	344
		5	49 bp	5076	5876	9213	66	6356	4024	105	303
		6	47 bp	7997	6176	10528	2	7950	4699	1545	115
		7	47 bp	8905	4445	4733	372	4623	7236	5201	825
		8	45 bp	8034	7013	12333	288	3741	2784	1560	123
		9	43 bp	10339	9958	11542	317	6639	144	1090	313
		10	42 bp	3795	8743	325	231	4180	9663	2555	613
	18	1	49 bp	11170	738	2220	302	4425	1877	2856	438

		2	42 bp	6158	8193	5063	482	4685	9077	383	218
		3	44 bp	834	7893	7977	36	4537	8933	1533	553
		4	42 bp	7818	9977	9208	194	7179	7885	1539	924
		5	43 bp	222	174	5245	116	7804	590	5165	261
		6	47 bp	6805	471	10202	423	6960	687	1219	246
		7	46 bp	2635	8931	5842	148	797	4436	3624	901
		8	44 bp	7292	5386	5877	162	5938	739	4825	129
		9	39 bp	6625	8779	3025	263	3379	2138	5074	221
		10	53 bp	5392	3523	12133	262	7126	1556	1181	672
7	19	1	53 bp	6877	5698	12542	307	6441	6764	4726	695
		2	57 bp	8955	7444	8873	349	4087	7929	4863	83
		3	57 bp	2552	7025	6844	304	2025	516	1840	404
		4	65 bp	8365	7393	11074	473	1925	6736	3451	812
		5	54 bp	4960	9860	5642	180	265	4358	5300	515
		6	63 bp	10486	6958	2825	444	1368	456	576	842
		7	50 bp	9420	6508	1240	70	2790	345	3691	112
		8	61 bp	3475	2325	5473	31	3012	878	2228	488
		9	46 bp	1261	1119	49	430	7588	8795	3954	709
		10	45 bp	2799	6316	1289	154	1957	120	1837	548
	20	1	47 bp	8721	477	10361	246	4760	1593	1068	429
		2	46 bp	9752	3392	8791	38	7352	110	4251	299
		3	53 bp	8610	2781	12125	125	1337	8843	1069	598
		4	57 bp	6037	2227	9959	264	1027	370	2441	333
		5	59 bp	8354	3352	7365	444	6751	3408	1547	62
		6	67 bp	7953	5705	9924	450	3504	4976	3424	513
		7	48 bp	1132	1680	5685	449	6702	6918	2332	221
		8	61 bp	1885	8029	10444	142	2692	1843	1402	561
		9	58 bp	6895	5781	10460	299	3060	9159	3726	498
		10	58 bp	7447	3280	424	402	6863	8890	4104	112
	21	1	50 bp	4037	5964	4468	285	6734	4947	3687	79
		2	44 bp	4042	5553	6899	394	3389	8248	2249	740
		3	53 bp	11826	4335	5257	216	5088	5061	4053	580
		4	63 bp	2676	5673	4951	60	2974	4199	4434	215
		5	47 bp	1332	7646	108	15	531	8612	1378	146
		6	44 bp	4668	7811	10643	156	4776	7300	2560	396
		7	55 bp	11017	4947	5794	393	349	8245	1345	74
		8	60 bp	5844	3874	2322	95	4248	2137	4013	822
		9	60 bp	9570	5753	6027	347	4617	6025	3947	775
		10	60 bp	11875	613	10240	353	3950	1061	2319	183
8	22	1	67 bp	924	2760	12475	286	7506	1327	2512	641
		2	56 bp	9716	5090	2537	163	554	9354	5242	331
		3	71 bp	4725	4526	4008	148	6287	201	1437	510
		4	76 bp	2471	1170	3233	354	2733	8194	562	275
		5	89 bp	11146	4130	7043	42	6696	3260	3084	465

		6	80 bp	5436	3314	6520	123	3491	1466	2052	586
		7	65 bp	10633	4582	6597	458	5154	9073	3357	893
		8	45 bp	4652	185	1923	146	611	1296	5298	367
		9	52 bp	5637	6612	6516	46	7471	3191	5659	769
		10	93 bp	3978	8361	1583	436	3972	2035	5375	866
	23	1	53 bp	1914	9032	8176	113	5431	8737	3185	620
		2	66 bp	235	3650	5379	393	7078	2440	5428	167
		3	66 bp	5696	9488	9506	126	2247	1295	4697	753
		4	50 bp	9786	362	3026	429	1576	4594	2480	706
		5	67 bp	3269	756	5395	360	7985	7554	4589	595
		6	72 bp	11546	4413	7085	204	132	599	474	970
		7	59 bp	8152	8801	8151	329	771	9245	1386	642
		8	48 bp	1523	5579	10276	370	3313	7421	4288	473
		9	75 bp	10583	9191	8579	365	3055	6210	4995	65
		10	71 bp	4613	7274	4678	43	2149	3555	2367	803
	24	1	58 bp	4781	3799	3958	15	7928	3503	334	524
		2	84 bp	695	3213	4264	141	1434	7008	5745	665
		3	78 bp	2020	2307	9222	123	5037	2832	1290	716
		4	79 bp	10257	6674	11820	248	2144	3253	1595	701
		5	142 bp	5127	7118	4224	171	1006	6816	1841	968
		6	56 bp	10394	2973	3013	225	5932	4102	5707	874
		7	110 bp	11577	9843	11074	451	2558	2795	3640	161
		8	94 bp	2931	2899	7981	469	4105	8061	434	685
		9	121 bp	11025	2308	7844	62	1302	1278	1979	717
		10	61 bp	11665	4645	1167	299	2913	6378	58	330
9	25	1	50 bp	2611	5360	798	190	1955	2954	5256	611
		2	52 bp	8145	653	10612	22	6466	5404	3650	713
		3	52 bp	2999	8423	2184	294	919	9514	1180	319
		4	77 bp	6869	8643	3957	139	1580	5591	5615	585
		5	52 bp	11278	8513	3673	470	1145	1834	3945	579
		6	70 bp	2683	1970	4057	396	6714	7209	1735	513
		7	52 bp	7437	6122	2444	389	7403	1471	3289	567
		8	53 bp	10485	2095	4755	356	4851	7901	1945	486
		9	53 bp	2575	1993	1246	473	7199	7178	4764	882
		10	79 bp	10874	435	6861	201	4697	7724	5315	258
	26	1	82 bp	11466	1960	3421	326	4727	2162	3707	87
		2	100 bp	10596	1283	4427	386	2700	7150	1343	306
		3	104 bp	11004	3047	5748	286	3046	6976	4678	741
		4	88 bp	3198	6660	10094	292	6306	9337	2673	948
		5	100 bp	10564	2106	8314	398	4880	6696	2435	319
		6	104 bp	2795	5604	1644	218	6354	1013	3326	916
		7	52 bp	11773	5721	3328	415	6462	8335	4387	460
		8	76 bp	8680	5447	6190	354	6508	7590	2342	421
		9	105 bp	1412	3596	3068	348	101	2792	5118	907

		10	93 bp	11651	5854	7934	139	1282	5891	517	962
	27	1	47 bp	11087	4995	10216	456	2188	8467	1902	418
		2	43 bp	2588	8753	7594	85	2007	5547	39	502
		3	47 bp	6523	6766	11023	315	2857	1221	3164	220
		4	48 bp	1094	7729	885	346	3096	2343	830	801
		5	40 bp	2644	2182	9666	209	3962	4671	1776	111
		6	56 bp	11192	3638	2307	306	1348	8218	3063	530
		7	45 bp	6452	3060	1896	193	1661	8793	4210	103
		8	53 bp	5096	2552	9588	479	7627	8118	4133	780
		9	45 bp	9494	2672	312	142	5067	8595	1588	426
		10	66 bp	11091	9746	7869	458	2276	4046	4328	912
10	28	1	49 bp	1840	7774	10705	258	3914	3016	356	824
		2	51 bp	10835	6907	884	174	194	5082	661	698
		3	53 bp	7198	4249	1557	390	6152	372	4928	120
		4	55 bp	1054	629	6865	214	3639	5897	122	338
		5	53 bp	396	825	7036	400	493	3366	3836	448
		6	51 bp	4132	7159	9631	81	3230	6580	5736	598
		7	51 bp	7312	5735	2069	58	1752	8243	4139	552
		8	52 bp	4465	3615	1045	267	5566	8146	4501	157
		9	52 bp	164	2562	7552	93	1703	3100	3105	281
		10	49 bp	1775	6061	10902	289	4659	7989	1247	123
	29	1	55 bp	7102	6214	2120	45	2992	4912	781	304
		2	58 bp	825	2600	1512	410	7652	4140	4884	438
		3	62 bp	10585	7545	1135	225	5227	2779	5802	312
		4	57 bp	11757	719	5967	18	7802	3222	4456	527
		5	60 bp	1195	6933	1969	104	4158	8647	1793	511
		6	67 bp	9939	98	224	304	2082	822	3649	597
		7	37 bp	9569	7841	3553	190	1029	6781	5722	398
		8	60 bp	2386	5685	7445	408	4024	6185	5439	661
		9	75 bp	9274	210	8945	138	6684	3951	1837	388
		10	78 bp	2367	1189	5731	105	5353	886	2928	94
	30	1	49 bp	10181	7306	3829	404	4755	7106	4627	404
		2	19 bp	10104	7614	862	51	2765	1888	1782	674
		3	48 bp	9978	2471	8487	294	4610	2700	2070	816
		4	43 bp	5157	1240	9678	148	2199	4433	5343	130
		5	43 bp	9588	1577	2247	361	5890	6558	3062	976
		6	46 bp	8545	3354	8587	479	7782	2957	5461	702
		7	51 bp	10077	4858	6410	99	155	3111	2175	537
		8	60 bp	6102	2352	3845	427	5131	4443	708	146
		9	37 bp	2196	9092	2453	419	2568	6560	3907	255
		10	44 bp	2088	7337	5626	235	7879	8182	2862	810
11	31	1	77 bp	8214	5348	12480	136	1177	6291	1149	362
		2	87 bp	9461	7028	6868	472	3892	4026	3663	497
		3	64 bp	7906	3025	11008	129	5890	2162	1089	944

		4	62 bp	7171	7886	9440	120	1673	4568	3281	749
		5	77 bp	9247	2226	12220	314	1050	3010	4043	176
		6	69 bp	3183	6305	11670	214	6574	3121	1394	781
		7	104 bp	4352	8119	3586	233	6977	6475	364	185
		8	61 bp	2235	3737	12538	75	4313	421	5142	524
		9	71 bp	3913	9538	3282	105	6398	8782	4797	176
		10	66 bp	9576	9141	7104	200	3579	7255	3009	474
	32	1	84 bp	4536	5638	1648	227	2578	1469	715	136
		2	57 bp	3752	8193	1093	161	5847	5584	2888	735
		3	71 bp	7188	4476	8013	164	5986	5668	3299	721
		4	52 bp	2061	1621	7022	22	6920	7216	3967	200
		5	72 bp	5585	2989	2350	141	5014	1304	5650	365
		6	61 bp	10256	2127	7645	384	415	1461	238	909
		7	43 bp	8657	8597	9681	131	2488	4430	1255	707
		8	59 bp	3123	7704	2035	279	1672	5651	486	395
		9	55 bp	11593	6355	4124	39	5220	8999	2367	403
		10	53 bp	1024	9970	12295	397	7547	3340	1059	104
	33	1	56 bp	11537	5356	11784	206	2223	4226	4718	864
		2	67 bp	8727	5156	7151	211	5746	7431	5311	866
		3	59 bp	948	6058	9846	476	5925	5413	3593	899
		4	56 bp	8979	6454	2126	356	3590	8291	2968	309
		5	75 bp	7098	7970	3043	204	4916	592	4943	832
		6	94 bp	5044	8260	8530	297	7344	5544	3684	95
		7	79 bp	8872	3517	11105	314	7575	7392	499	548
		8	68 bp	8999	6112	1540	180	219	4896	3273	560
		9	64 bp	6035	2471	2343	105	1950	9459	2933	352
		10	74 bp	6972	9396	1128	104	4385	8927	253	63
12	34	1	83 bp	10819	5506	6852	56	2733	330	1746	314
		2	98 bp	11181	1471	2792	191	7723	1316	2342	146
		3	92 bp	5278	5408	5243	71	5818	208	1516	936
		4	62 bp	54	7236	1321	481	1366	5839	1109	777
		5	65 bp	10845	8977	973	331	4485	1214	831	466
		6	102 bp	3675	1338	1439	163	1739	324	4910	413
		7	97 bp	8679	7807	6824	227	1595	9243	5672	879
		8	103 bp	76	1767	7540	439	7546	7225	1094	451
		9	96 bp	11627	1895	3239	54	6641	4557	3261	966
		10	109 bp	9452	4018	2309	350	5791	6379	5757	82
	35	1	86 bp	6478	6000	2890	431	4239	6428	4487	556
		2	84 bp	2874	4652	7567	460	2632	7633	3131	519
		3	100 bp	6798	4486	12122	268	5331	3136	3544	910
		4	112 bp	2751	5119	11393	50	2101	4921	5052	249
		5	97 bp	905	9279	282	268	5867	2201	4218	815
		6	97 bp	8154	2317	9014	200	5109	7057	4339	528
		7	115 bp	5364	2861	11219	160	2461	5422	1625	176

		8	98 bp	2225	1842	4914	55	7682	4319	1738	652
		9	109 bp	2268	912	7210	34	6807	2701	618	619
		10	81 bp	7651	4034	7701	133	5059	3214	5286	749
	36	1	56 bp	3161	4660	2516	474	3849	2812	4094	821
		2	112 bp	10547	6902	6145	290	7065	7269	4116	744
		3	99 bp	7026	6036	9176	228	4929	9378	585	800
		4	101 bp	2810	3815	8837	362	5160	2666	1932	137
		5	80 bp	6365	7484	4784	279	7465	8373	5493	806
		6	100 bp	3828	9188	100	347	6240	4595	44	944
		7	112 bp	711	367	7267	273	908	246	3766	905
		8	115 bp	9472	7433	7752	369	68	8828	5619	312
		9	80 bp	4094	8443	6876	170	7184	4930	1584	89
		10	121 bp	10446	2003	11057	27	2842	1352	5022	508
13	37	1	99 bp	9311	8451	2268	278	497	8085	4149	378
		2	83 bp	2148	7165	3808	262	1885	1801	4693	903
		3	108 bp	519	6892	10050	274	6121	6247	3914	57
		4	60 bp	4408	1699	11827	204	3141	9422	5112	804
		5	95 bp	9007	8552	8822	375	7575	2019	1530	311
		6	111 bp	7802	9079	1016	381	816	3008	3417	663
		7	94 bp	810	9057	3958	64	3901	8782	3850	602
		8	100 bp	10313	8761	4915	65	578	9237	561	488
		9	133 bp	8935	4015	4120	229	400	5066	529	916
		10	103 bp	10190	8085	6413	377	26	5097	2557	251
	38	1	118 bp	3062	1128	2236	358	3029	1344	876	262
		2	110 bp	1377	3449	199	264	5078	6783	4169	549
		3	91 bp	958	5493	10933	273	863	1465	3623	804
		4	74 bp	11080	7266	11238	248	3273	6319	1592	90
		5	64 bp	2594	2647	3116	342	1546	7480	5819	815
		6	69 bp	223	7605	7016	252	2347	9607	578	682
		7	80 bp	3195	9551	6642	254	5851	1687	3872	960
		8	88 bp	5493	5790	7359	437	7570	9197	231	764
		9	70 bp	5759	6469	10525	471	5207	8954	5157	305
		10	75 bp	5414	3426	6174	134	5463	7681	3462	437
	39	1	42 bp	4397	1195	7034	485	2369	1126	416	148
		2	110 bp	8399	2126	3038	364	6194	8101	2447	228
		3	116 bp	4262	8125	6911	314	838	3052	2632	125
		4	127 bp	4388	3660	7919	86	63	8682	2797	193
		5	116 bp	7236	9679	3956	398	6372	3417	5217	550
		6	106 bp	2858	5763	6463	172	3886	9085	4272	815
		7	116 bp	8678	16	1516	43	4730	8232	5085	636
		8	89 bp	10768	9517	1941	312	1999	8558	3044	504
		9	114 bp	9920	4230	4886	403	3193	3104	5215	701
		10	95 bp	8196	2025	6771	209	7566	4144	5048	607
14	40	1	50 bp	11685	7661	6425	394	5412	2295	2803	912

		2	62 bp	2964	3307	3020	435	2284	2124	2553	446
		3	63 bp	9847	2436	5288	147	4138	9145	3433	880
		4	51 bp	6215	3743	7004	175	7840	3651	5635	632
		5	53 bp	3737	3857	3565	361	3246	1336	5558	684
		6	48 bp	9099	4607	7507	29	4547	4847	2014	296
		7	49 bp	3171	8171	5255	112	2819	6234	4918	412
		8	42 bp	8561	3762	3088	467	736	7625	3107	604
		9	51 bp	9273	6958	11664	347	7578	5406	3782	211
		10	43 bp	155	3645	7694	183	2415	2960	4736	866
	41	1	44 bp	1719	5462	3942	169	425	8335	5040	70
		2	55 bp	2653	2143	9116	58	4325	2635	3050	955
		3	86 bp	4416	5475	3057	447	3611	8019	1628	206
		4	39 bp	6895	387	6829	158	5769	7594	4915	50
		5	44 bp	11514	4971	5786	93	728	9399	5446	992
		6	55 bp	3957	9861	12084	298	1634	3061	961	354
		7	50 bp	7753	5636	8811	146	5640	1826	259	118
		8	50 bp	5289	3549	4200	350	7333	9071	996	524
		9	48 bp	2034	3030	6720	304	3927	3954	3131	427
		10	46 bp	4427	5199	4396	197	2711	4599	621	569
	42	1	56 bp	6304	4840	10657	390	3450	429	312	258
		2	43 bp	8689	6460	11733	53	2480	6645	138	542
		3	56 bp	7840	8809	7723	139	5094	1824	676	838
		4	57 bp	11086	366	4722	265	3748	9242	5629	141
		5	58 bp	782	9726	10933	428	7965	7343	4175	313
		6	50 bp	7795	975	7491	401	1030	253	455	453
		7	78 bp	9065	7398	8603	349	4543	666	2838	926
		8	58 bp	4503	9482	2784	152	2908	6261	3763	862
		9	77 bp	2156	1811	6499	114	840	1883	1407	240
		10	44 bp	6079	1363	7366	371	1886	6935	2456	111

Appendix F: Primers used for p.L264R validation PCR.

Nested PCR Round 1:

p.L264R FwN: 5’ – CCTTACTGCCTCCTCTTGCTTCT – 3’

p.L264R Rv2: 5’ – CAGGCTAGGCTAAGCTATGA – 3’

Nested PCR Round 2:

p.L264R Fw: 5’ – ATCCTGAGTAGTGGCAGTTG – 3’

p.L264R Rv: 5’ – GTTGGTGTTCTGAAGTTAGT – 3’